COEXISTENCE OF QUINOLONE RESISTANCE AND EXTENDED SPECTRUM BETA LACAMASE PRODUCTION IN CLINICAL ISOLATES OF *ESCHERICHIA COLI* IN A TERTIARY CARE CENTRE

DISSERTATION SUBMITTED TO

In partial fulfillment of the requirement for the degree of

DOCTOR OF MEDICINE IN MICROBIOLOGY

(Branch IV) M. D. (MICROBIOLOGY)

of

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CHENNAI- 600032

DEPARTMENT OF MICROBIOLOGY

TIRUNELVELI MEDICAL COLLEGE

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BONAFIDE CERTIFICATE

This is to certify that the dissertation entitled **"COEXISTENCE OF QUINOLONE RESISTANCE AND EXTENDED SPECTRUM BETA LACAMASE PRODUCTION IN CLINICAL ISOLATES OF *ESCHERICHIA COLI* IN A TERTIARY CARE CENTRE"** submitted by **Dr.V. INDUMATHI** to The Tamilnadu Dr. M.G.R Medical University, Chennai, in partial fulfillment of the requirement for the award of M.D. Degree Branch – IV (Microbiology) is a bonafide research work carried out by her under direct supervision & guidance.

Head of the Department,
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CERTIFICATE

This is to certify that the Dissertation **"COEXISTENCE OF QUINOLONE RESISTANCE AND EXTENDED SPECTRUM BETA LACAMASE PRODUCTION IN CLINICAL ISOLATES OF *ESCHERICHIA COLI* IN A TERTIARY CARE CENTRE**" presented herein by **Dr.V.INDUMATHI** is an original work done in the Department of Microbiology,Tirunelveli Medical College Hospital, Tirunelveli for the award of Degree of M.D.(Branch IV) Microbiology under my guidance and supervision during the academic period of 2014-2017.

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DECLARATION

I, **Dr. V. INDUMATHI** declare that, I carried out this work on **"COEXISTENCE OF QUINOLONE RESISTANCE AND EXTENDED SPECTRUM BETA LACAMASE PRODUCTION IN CLINICAL ISOLATES OF *ESCHERICHIA COLI* IN A TERTIARY CARE CENTRE**" at the Department of Microbiology, Tirunelveli Medical College, I also declare that this bonafide work or a part of this work was not submitted by me or any others for any award, degree, or diploma to any other University, Board, either in India or abroad.

This is submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the rules and regulations for the M.D Degree examination in Microbiology.

Place: Tirunelveli **Dr. V. Indumathi**

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PROTOCOL TITLE: COEXISTENCE OF QUINOLONE RESISTANCE AND EXTENDED SPECTRUM BETA LACTAMASE PRODUCTION IN CLINICAL ISOLATES OF ESCHERICHIA COLI IN A TERTIARY CARE CENTRE

PRINCIPAL INVESTIGATOR: DR.V.INDUMATHI, MBBS.,

DESIGNATION OF PRINCIPAL INVESTIGATOR POST GRADUATE IN MICROBIOLOGY
DEPARTMENT & INSTITUTION: TIRUNELVELI MEDICAL COLLEGE , TIRUNELVELI

Dear , Dr.V.Indumathi, MBBS., The Tirunelveli Medical College Institutional Ethics Committee (TIREC) reviewed and discussed your application during the IEC meeting held on 10.12.15.

THE FOLLOWING DOCUMENTS WERE REVIEWED AND APPROVED

1. TIREC Application Form ☐
2. Study Protocol ☐
3. Department Research Committee Approval ☐
4. Patient Information Document and Consent Form in English and Vernacular Language ☐
5. Investigator's Brochure ☐
6. Proposed Methods for Patient Accrual Proposed ☐
7. Curriculum Vitae of the Principal Investigator ☐
8. Insurance /Compensation Policy ☐
9. Investigator's Agreement with Sponsor ☐
10. Investigator's Undertaking ☐
11. DCGI/DGFT approval ☐
12. Clinical Trial Agreement (CTA) ☐
13. Memorandum of Understanding (MOU)/Material Transfer Agreement (MTA) ☐
14. Clinical Trials Registry-India (CTRI) Registration ☐

THE PROTOCOL IS APPROVED IN ITS PRESENTED FORM ON THE FOLLOWING CONDITIONS

1. The approval is valid for a period of 2 year/s or duration of project whichever is later
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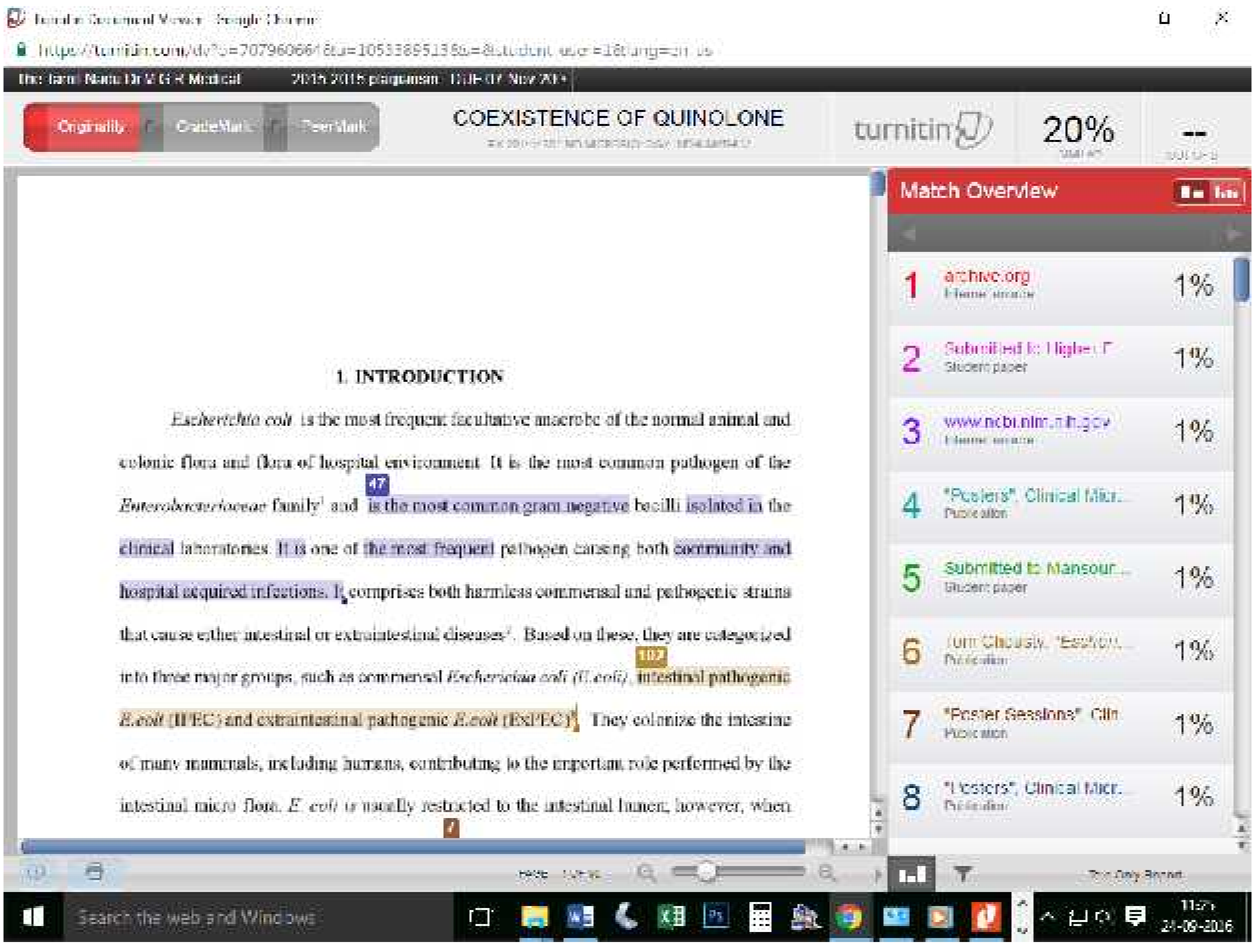
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1. INTRODUCTION
Escherichia coli is the most frequent facultative anaerobe of the normal animal and colonic flora and flora of hospital environment. It is the most common pathogen of the Enterobacteriaceae family[1] and is the most common gram negative bacilli isolated in the clinical laboratories. It is one of the most frequent pathogen causing both community and hospital acquired infections. It comprises both harmless commensal and pathogenic strains that cause either intestinal or extraintestinal diseases[2]. Based on these, they are categorized into three major groups, such as commensal Escherichia coli (E.coli), intestinal pathogenic E.coli (IPEC) and extraintestinal pathogenic E.coli (ExPEC)[3]. They colonize the intestine of many mammals, including humans, contributing to the important role performed by the intestinal micro flora. E. coli is usually restricted to the intestinal lumen; however, when
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LIST OF ABBREVATIONS

E.coli	*Escherichia coli*
IPEC	Intestinal pathogenic *Escherichia coli*
ExPEC	Extraintestinal pathogenic *Escherichia coli*
UTI	Urinary tract infection
ESBL	Extended Spectrum Beta Lactamase
CLSI	Clinical Laboratory Standards Institute
CDDT	Combined Disk Diffusion Test
DDST	Double Disk Synergy Test
E test	Epsilometer test
AMP	Adenosine Mono Phosphate
ADP	Adenosine Di Phosphate
MIC	Minimum Inhibitory Concentration
PCR	Polymerase chain reaction
CFU	Colony Forming Unit
MHA	Muller Hinton Agar
ATCC	American Type Culture Collection

CSF	Cerebrospinal Fluid
μg	microgram
DNA	Deoxy Ribonucleic Acid
IC	Internal Control
CT	Cross Threshold
PMQR	Plasmid mediated Quinolone resistance

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1. INTRODUCTION

Escherichia coli is the most common pathogen of the *Enterobacteriaceae* family[1]and most frequent gram negative bacilli isolated in the clinical laboratories. It is the major pathogen causing both community and hospital acquired infections. It comprises both harmless commensal and pathogenic strains that cause either intestinal or extraintestinal diseases[2]. Based on these, they are categorized into three major groups, such as commensal *Escherichia coli (E.coli)*, intestinal pathogenic *E.coli* (IPEC) and extraintestinal pathogenic *E.coli* (ExPEC)[3]. They colonize the intestine of many mammals, including humans, contributing to the important role performed by the intestinal micro flora. *E. coli is* usually restricted to the intestinal lumen; however, when these bacteria are introduced to other sites following trauma or surgical procedures or in immunocompromised individual even commensal, “nonpathogenic” strains of *E. coli* can cause infection[2]. In addition, there is an increased rate of vaginal colonization with *E.coli* among the postmenopausal women and in women who use diaphragms and/or spermicidal agents for contraception. This extended niche is an important predisposing factor that allows subsequent extraintestinal infections to occur.

ExPEC is the most frequent cause of community and hospital acquired urinary tract infections (including infections of the kidney) accounting for more than 80% of Urinary Tract Infection (UTI) [4]. It is the most frequent cause of bloodstream infection at all ages and is also associated with intra-abdominal infections such as peritonitis, and with skin and soft tissue infections due to multiple microorganisms. It is one of the most common causes of meningitis in neonates and is one of the leading causative agents of food borne infections worldwide[2].

Infections with *E. coli* usually originate from the person affected (auto-infection), but strains with a particular resistance or disease-causing properties can also be transmitted from animals, through the food chain or between individuals by person-to-person contact, or contact with environments or fomites contaminated with fecal material[2].

Trimethoprim/sulfamethoxazole, quinolones, beta lactams with or without inhibitors, nitrofurantoin and fosfomycin are the most commonly used antibacterial drugs in the treatment of infection caused by *E.coli.* An important aspect of treating infection caused by *E.coli* is its increase in resistance to antibiotics[5].

Betalactams are group of antibiotics that inhibits bacterial cell wall synthesis. The mechanism of resistance produced to these antibiotics is chromosomally mediated or plasmid mediated. Extended spectrum–beta lactamase (ESBL) enzymes are plasmid mediated, capable of hydrolyzing many beta-lactam antibiotics including 3rd generation cephalosporins and monobactams and are inhibited by beta-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam[6].

The first ESBL was identified in Germany in 1983; since then, over 200 variants of the clavulanic acid-inhibited form of the enzyme have been described worldwide[7]. Prevalence of ESBL production in gram negative bacilli varies widely among different geographical region and in different clinical settings[8, 9, 10]. Plasmids responsible for ESBL production tend to be large (80 Kb or more in size) and frequently encode for resistance to other class of antimicrobials also, thus limiting the choice of antimicrobials available for the treatment of infections[11]. The most common coresistance found in ESBL producing organisms are aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol and sulfamethoxazole-trimethoprim[12].

Quinolones and fluoroquinolones (FQ) are potent antimicrobial agents, used in a wide spectrum of infections such as UTI, respiratory tract infections, skin and soft tissue infections, prostatitis, sexually transmitted diseases and bone and joint infections[13]. Quinolone resistance among *Enterobacteriacae* has increased significantly since their use began in the 1980s. Extensive use of these drugs leads to increasing level of resistance to quinolones. The mechanism of action of quinolone is owing to inhibition of bacterial DNA synthesis. Multiple chromosomal mutation affecting the target enzymes DNA gyrase and topoisomerase IV, decreased uptake of drug due to the loss of a membrane-bound porin or via efflux pump mechanism contributes to resistance production[14].

Plasmid mediated quinolone resistance gene *qnr* was first identified in 1998 in a clinical isolate of *klebsiella pneumoniae*[15]. The plasmid encoded protein responsible for resistance is termed Qnr. It has now been found worldwide. These plasmids protect DNA gyrase and topoisomerase IV from the inhibitory activity of the quinolones. A new mechanism of transferable quinolone resistance was reported recently, is due to enzymatic inactivation of certain quinolones. The variant of *aac(6')-Ib-cr* encodes an aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin by N-acetylation of its piperazinyl amine[15]**.** Plasmid mediated quinolone resistance gene is often located with an ESBL gene thus facilitating transfer of both quinolone resistance and ESBL among enterobacteriaceae[13]. Among the plasmid mediated quinolone resistant gene *aac(6')-Ib-cr, qnrA and qnrB are* the most common gene harboured[13,14].

The increasing trends of antibiotic resistance among *E.coli* isolates make a major growing public health problem worldwide. The risk factors for multi drug resistance are prolonged exposure to antibiotics, increased length of stay in ward or in Inttesive Care Unit, severity of illness, malignancy, age more than 65 years, intravenous devices and cathter[16]. It also poses a great impact to efficacy of antibiotics. When these resistant bacteria spread to the community, it creates major difficulty in infection control issues. Control of antibiotic resistance is important primarily because resistance limits treatment options, sometimes to less effective or more toxic therapies. Excess cost or length of hospital stay, increase in morbidity and mortality has been found to be associated with infection due to these pathogens. Thus early detection of multi drug resistance is critical in preventing the spread of these drug resistant bacteria.

There are different methods to detect phenotypically the production of the ESBL and quinolone resistance in the formulated guidelines from the CLSI. The various phenotypic methods that are available to detect the ESBL resistance includes, combined disc diffusion test (CDDT), double disc synergy test (DDST), ESBL E-test (Epsilomer test) and broth dilution test. Disc diffusion test and MIC (Minimum Inhibitory Concentration) determination are available for quinolone resistance detection. The molecular detection of the ESBL and quinolone resistance using PCR (Polymerase Chain Reaction) is the standard test.

Thus this study is carried out to detect the presence of quinolone resistance among ESBL producing *E.coli* and the susceptibility pattern of these strains to other antibiotics prescribed in our hospital.

2.AIMS AND OBJECTIVES

- To assess the antibiotic susceptibility pattern in *Escherichia coli* isolates in Tirunelveli Medical College.
- To detect the ESBL production in *Escherichia coli* isolates by phenotypic methods.
- To detect the quinolone resistance in *Escherichia coli* by phenotypic methods.
- To detect the coexistence of quinolone resistance and ESBL production among the *Escherichia coli* isolates.
- To detect the plasmid mediated quinolone resistance gene *qnrA, qnrB* and *aac(6')-Ib-cr* among the quinolone resistance ESBL producing isolates of *Escherichia coli.*

3. REVIEW OF LITERATURE

3.1 HISTORY[1]

Escherichia coli was first isolated from faeces of an infant by the German pediatrician Theodore Escherich, while he was studying the intestinal flora of infants. He described the colon bacillus under the name *Bacterim coli commune* in 1885. Castellani and Chalmers defined the genus *Esherichia*, and the type species was *Escherichia coli*.

3.2 MORPHOLOGY:

Escherichia coli are gram-negative, nonsporing, straight rod-shaped bacillus which are usually (80% strains) motile by peritrichous flagella. They vary in size between 1-3 µm × 0.4-0.7 µm. They are arranged singly or in pairs Strains which are responsible for extra intestinal infections are usually capsulated. The capsule is polysaccharide in nature. The fimbriae are present on almost 80% of strains. In most of the strains these are of type 1[1].

3.3 CULTURAL CHARACTERISTICS:

E.coli is an aerobe and facultative anaerobe. This organism can grow easily on ordinary medium and does not have complex nutritional requirements. It grows over a wide range of temperature (15°C-45°C) but 37°C is the optimal temperature.

In nutrient agar, colonies are large, 1-1.5 mm in diameter, thick grayish, smooth, circular, glossy and translucent. Colonies are easily emulsifiable. Colonies are of two type, glistening, smooth (S) , or dry wrinkled, rough (R) colonies depending on the state of lipopolysaccharide of the outer membrane.

In liquid media, S forms show homogenous turbid, whereas R forms sediment on the bottom of the test tubes. In heavily fimbriate strains, it forms pellicle on the surface of the liquid media. On MacConkey's agar, colonies are pink in colour due to lactose fermentation. On blood agar, the colonies of some strains are surrounded by zone of haemolysis.

3.4 BIOCHEMICAL REACTIONS:

They are catalase positive, ferments most of the carbohydrate with production of acid and gas. They produce indole, fail to hydrolyze urea. They do not utilize citrate. Methyl red reaction is positive and Voges – Proskauer is negative. Nitrates are reduced to nitrites.

phenylalanine is not deaminated, gelatin is not liquefied, and gluconate is not oxidized. Most strain decarboxylase lysine. Most strains of this species ferment lactose and give a positive O-nitrophenyl-β-D-pyranoside (ONPG) reaction.

On Triple sugar iron agar acid slant and acid butt are produced with gas and H_2S is not produced.

3.5.VIRULENCE FACTORS

3.5.1 K antigens.

These are the surface polysaccharides that protect the bacteria from phagocytosis. The K1 strain of *E. coli* is a major cause of neonatal meningitis. There are certain K types which are more commonly associated with infections in the urinary tract. These include K1, K2, K3, K5, K12 and K13.

3.5.2 Colonisation factor.

Fimbriae (or pili), present on the surface of many strains of *E. coli* that play main role in pathogenesis because of their adhesive nature. It also helps the enterotoxin producing *E. coli* to deliver their toxin close to the host cell. Four colonization factor antigens (CFA) have been detected from enterotoxigenic strains that cause disease in human beings. These have been designated as CFA/I, CFA/II, CFA/III and CFA/IV. These are distinguished on the basis of their antigenicity as well as ability to agglutinate human or bovine erythrocytes.

3.5.3 Endotoxin:

Most of the activities of endotoxin exist in in lipid A moiety They are responsible for the pyrogenicity, utilisation of complement, consumption of coagulation factors and shock. Injection of large doses of LPS causes hypotension, irreversible shock and death.

3.5.4 Enterotoxin:

Enterotoxins are important in the pathogenesis of diarrhea. Three different type of enterotoxins are produced. They are heat stable enterotoxin (ST) and heatlabile enterotoxin (LT) and shiga-like toxin (SLT) also known as verotoxin(VT).

3.5.5 Heat Labile Enterotoxin (LT)

LT is structurally and functionally similar to cholera toxin and has a molecular weight of 86000 daltons. It is protein in nature. It is composed of two peptide fragments A and B. Fragment B is the binding fragment that has five subunits.it binds to GM1 ganglioside receptors present on the intestinal epithelium after which A fragment, the active fragment is internalized and cleaved into A1 and A2 peptides. Fragment A2 helps in tethering A and B subunits together. Fragment A1 is the active fragment, causes ADP ribosylation of G protein which up regulates the activity of adenylate cyclase, results in the intracellular accumulation of cyclic AMP. Increased cyclic AMP leads to the outflow of water and electrolytes into the gut lumen, with consequent diarrhea.

3.5.6 Heat Stable Enterotoxin (ST)

ST enterotoxin has a low molecular weight and is poorly immunogenic. Two different types have been recognized. These are ST-I and STII. ST-I binds to the guanylate cyclase C which leads to increased production of cyclic guanosine monophosphate (cGMP). cGMP increases the accumulation of fluid in the gut lumen leading to diarrhea.

3.5.7 Vero cytotoxin.

This is also known as 'Shiga-like toxin' (SLT) because it has similar biological properties, physical characters and antigenicity. It is of two types VT1 and VT2, the former is neutralised by antishiga toxin whereas the latter resists this neutralisation. It has two fragments A and B. Fragment B binds to globotrosyl ceramide receptor on intestinal epithelium. Fragment A is the active fragment. It inhibits protein synthesis by inhibiting 28S and 60S subunit ribosome.

3.5.8 Cytotoxin:

This is an enterotoxic substance which is similar to the one released by some shigellae. Some ExPEC strains release it and in association with EAF it is believed to cause diarrhoea.

3.6 CLINICAL MANIFESTATIONS:

E.coli is the most common pathogen encountered clinically and has been associated with various manifestations.

3.6.1 EXTRA INTESTINAL INFECTIOUS SYNDROME

3.6.1.1Urinary Tract Infection

The urinary tract is the most common site infected by ExPEC. UTI is the second most common infection responsible for hospitalization next only to the lower respiratory infection. *E. coli* is the most common pathogens and accounts for about 80% of all UTI syndrome. Latika J Shah**,** et al in their study showed that about 61.3% isolates of the urine are *E. coli*[21]. In various studies conducted by madhurendhra singh et al, manikandan et al, and a, sharma et al, the most common (54- 66%) isolate in urine were *E. coli*[22,23,24] .

3.6.1.2 Abdominal and pelvic Infectlon

The abdomen/pelvis is the second most common site of extra intestinal infection due to *E. coli*. It is the most common cause of both primary bacterial peritonitis and secondary bacterial peritonitis. It also causes diverticulitis, appendicitis, visceral abscesses, such as hepatic abscess.

It is the common pathogen isolated in spontaneous bacterial peritonitis patients. Safia Bibi, et al in her studies reported that *E.coli* was the predominant pathogen(65%) among ascitic fluid collected from spontaneous bacterial peritonitis[25].

Payal H Purohit, et al, 2014 in his study with 34 isolates from ascitic fluid concluded that the most common organisms isolated were *E. coli* (54.9%)[26].

Dimple kasana et al reported that the most common (30.7%) bacteria isolated from ascitic fluid were *E. coli*[27].

3.6.1.3 Wound And Soft Tissue Infections

E.coli is one of the most frequent organisms that cause infections of decubitus ulcers and occasionally to infections of wound of the lower extremity in diabetic patients and other hosts with neurovascular compromise. *E.coli* can also causes cellulitis or infections of burn sites and surgical wounds (accounting for -10% of surgical site infections).

A study by Sah et al on post-operative wound infections reported that the second most common (29.6%) bacteria isolated was *E.coli*[28]. *C*hauhan et al in a similar study concluded that *Pseudomonas aeruginosa* and *E.coli,* were the leading Gram-negative isolates in wound infections[29]. *B*asu S et al and Zubair.M.et al too reported *Pseudomonas* and *E.coli* to be the most commonly occurring gram negative pathogens in wound infections[30,31].

3.6.1.4 Meningitis

E.coli is one of the most common causes of neonatal meningitis, the other being group B *Streptococcus*. Most *E. coli* strains that cause neonatal meningitis possess the Kl capsular antigen. *E.coli* meningitis is uncommon after the neonatal period.

Liu Cu-Qing et al, 2014 reported *E. coli* to be the most common cause of neonatal meningitis[32].

3.6.1.5 Pneumonia

E.coli is an uncommon cause of pneumonia. Enteric gram negative bacteria accounts for only 1-3% of community-acquired pneumonia. However, they are the most common cause (60-70% of cases) of hospital-acquired pneumonia, particularly among postoperative and ICU patients. *E.coli* is usually the third or fourth most commonly isolated gram negative bacteria in hospital-acquired pneumonia, which accounts for 5-8%[33].

3.6.1.6 Bacteremia

E.coli bacteremia mainly arises from any infection at extra intestinal site. It may be community acquired or hospital acquired. The urinary tract infection is the most common source of *E.coli* bacteremia. The abdomen is the second most common source, accounting for 25% of bacteremia due to *E.coli*[33].

3.6.2 Intestinal Infections[34]:

Diarrhea: It is caused by six types of diarrheagenic *E.coli.*

1. Enteropathogenic *E.coli* (EPEC)- causes infantile diarrhea and occasionaly causes sporadic diaarhea in adults.
2. Enterotoxigenic *E.coli* (ETEC) is the most common cause of traveler's diarrhea causing 25-75% of cases. It causes watery diarrhea in infants and adults. Diarrhea is due to the production of heat labile and heat stable toxin.
3. Enteroinvasive *E.coli* (EIEC) causes ulceration of bowel, dysentery. It is not toxigenic but invasive. It invade epithelial cell due to the presence of Virulence Marker Antigen(VMA).

4. Enterohemorrhagic *E.coli* (EHEC)- O157:H7 is the most common serotype. It causes hemorrhagic colitis and hemolytic uremic syndrome (HUS). EHEC secretes a toxin called verocytotoxin or shiga-like toxin.
5. Enteroaggregative *E.coli* (EAEC) is so named because it adheres to HEp-2 cells in a distinct pattern, layering of the bacteria in a stacked-brick fashion. It causes persistent and acute diarrhea, especially in developing countries.
6. Diffusely adherent *E.coli* (DAEC), primarily causes disease in children aged 2-6 years.

3.7 ANTIMICROBIAL THERAPY:

Various groups of antimicrobial agents were used in the treatment of infections caused by *E.coli*. Earlier *E.coli* were susceptible to many antibiotics, however the situation has changed and there is an high prevalence of drug resistance among the *E.coli* isolates. This may be due to the increase in the irrational prescription of antibiotics, even for the community acquired infections leading to resistance to first generation Cephalosporins, Trimethoprim-Sulfomethoxazole. This results in the decrease in the cure rates leading to switch to alternate drugs such as Quinolones. This in turn accelerates the emergence of resistance to fluoroquinolones[34].

3.7.1 BETA – LACTAM ANTIBIOTICS

Beta lactam antibiotics are group of drugs that contain a common beta lactam ring in their structure. They differ from one another by the side chains attached to these ring[35].

Beta lactam antibiotics comprised of five different groups of antibiotics. They are Penicillins, Cephalosporins, Monobactams, Carbapenems and Cefamycins.

3.7.1.1 Penicillins[36]:

Penicillin was originally obtained from the fungus *Penicillium notatum*. Penicillin contains thiazoilidine ring fused with the beta lactam ring with aside chain attached at position 6. The various groups of penicillins are natural penicillins, semi-synthetic penicillins, penicillinase resistant penicillins, amino penicillin and extended-spectrum penicillins.

3.7.1.2 Cephalosporins[36]:

The Cephalosporins belongs to β-lactam antibiotics that are closely related to the penicillins. The first cephalosporin was obtained from *Cephalosporium acremonium*. The Cephalosporins consists of a β-lactam ring fused to a dihydrothiazine ring. Mechanisms of action of Cephalosporins are similar to the penicillins and have the same resistance mechanisms. However, they are more resistant to certain β-lactamases than the penicillins. Based on their bacterial susceptibility patterns and resistance to β-lactamases, cephalosporins are classified into five generations.

3.7.1.3 Monobactams:

Monobactams are group of beta lactam drugs with a monocyclic β-lactam ring. They are active against gram negative organisms and do not act on gram positive organisms and anaerobes.

3.7.1.4 Carbapenams

Carbapenems are synthetic β-lactam antibiotics in which the sulfur atom of the thiazolidine ring has been externalized and replaced by a carbon atom. Imipenem , Meropenem , Doripenem , and Ertapenem are the drugs of this group currently available.

3.7.1.5 Mechanism of Action:

Beta lactam antibiotics act by inhibiting the transpeptidase enzyme thereby inhibits the bacterial peptidoglycan synthesis which is a heteropolymeric component of the cell wall that gives stability to the organis. This leads to bacterial cell lysis and cell death.

3.7.2 β-Lactamase Inhibitors :

The molecules that inactivate the enzyme β-lactamase, and thereby protects the β - lactam antibiotics from destruction are called β-lactamase inhibitors. They are mostly active against plasmid-encoded β-lactamases that hydrolyze ceftazidime and cefotaxime. Clavulanic acid, sulbactam, tazobactam are the common β-lactamase inhibitors used against *Enterobacteriaceae* infections now a days.

3.7.3 QUINOLONES

Quinolones are synthetic antimicrobial agents developed in 1980s. Nalidixic acid was the first quinolone which was commonly used in the treatment of urinary tract infections. Newer quinolones have been synthesized by modifying the original two-ring quinolone (or naphthyridone) nucleus with different side chain substitutions[37].

3.7.3.1Mechanism of action:

Fluoroquinolones enter the bacterial cells by passive diffusion through porin channels. These drugs act on the enzyme DNA gyrase, a type II DNA topoisomerase enzyme and DNA topoisomerase IV. DNA gyrase A subunit is the main target of quinolones in gram-negative bacteria, whereas topoisomerase IV is the primary target in gram-positive bacteria[38]. Inhibition of these bacterial enzymes causes relaxation of the supercoiled DNA, leading to termination of bacterial DNA replication and interferes with cell division. In addition, the bacterial DNA gyrase inhibition also leads to extensive filamentation, vacuole formation and degradation of chromosomal DNA by exonucleases whose production is signaled by damaged DNA. The antibacterial activity of quinolones is reduced in the presence of low pH, urine, and divalent cations.

3.7.3.2 Classification:

Based on the time of their introduction into clinical practice and spectrum of activity, quinolones and fluroquinolones are grouped into different categories[36].The first generation includes nalidixic acid and cinoxacin. They are the oldest and least often used drugs. They have narrow spectrum of activity, poor tissue penetration and reach minimal serum concentration. They are used only in uncomplicated UTIs. The narrow spectrum quinolones are inactive against gram-positive cocci. Broad-spectrum (second-generation) fluoroquinolones are active against both gram-positive and gram-negative bacteria. They include ciprofloxacin, norfloxacin, ofloxacin, lomefloxacin, enoxacin and pefloxacin.

Gatifloxacin, gemifloxacin, and moxifloxacin make up a third group of fluoroquinolones (Expanded spectrum quinolones) with improved activity against gram positive organisms, particularly *S pneumoniae* and some *staphylococci*.

Extended spectrum (fourth generation) Sparfloxacin also has activity against few anaerobes, *chlamydia* and some *rickettssiae.*

3.7.4 AMINOGLYCOSIDES

It includes gentamicin, kanamycin, tobramycin, streptomycin, netilmicin, neomycin, and amikacin. Aminoglycosides bind the 30S ribosomal subunit, where they interfere with assembly of the functional ribosomal apparatus thereby inhibits protein synthesis.

3.7.5 OTHER ANTIMICROBIALS

.Macrolides, Chloramphenicol, lincosamides, streptogramin binds to the 50S ribosome and inhibits protein synthesis. Sulfonamides and trimethoprim inhibits folate synthetase and dihydrofolate reductase respectively and inhibits folic acid synthesis.

3.8 MECHANISM OF ANTIBIOTIC RESISTANCE[39]:

Gram-negative organism develops resistance to antimicrobial agents by five main methods. (i) Bacteria may carry genes that codes for enzymes that can inactivate antimicrobials. Such as β-lactamase enzyme produced by the bacteria hydrolyzes and inactivates the beta-lactam antibiotics. (ii) Bacteria can develop resistance by modifying the binding sites for antibiotics. (iii) Certain bacteria modify their cell membrane porins thereby preventing the antimicrobial from entering into the cell. (iv) Bacteria can express efflux pumps to actively transport antibiotics out of the cell and finally,(v) alternate metabolic pathways that can by-pass path inhibited by antibiotics. Resistance in Gram-negative bacteria may be intrinsic, or they may be acquired. Microorganism very often possesses various mechanisms to express the resistance towards almost all the available antibiotics and is often a combination of resistance mechanism like beta-lactamases, porin deletions and efflux pumps. The predominant mechanism of resistance is, however, the hydrolysis of the antibiotic by beta-lactamases.

3.8.1 Enzymatic – Beta-lactamases:

β-lactamases are the enzymes with serine proteases at their active-site. β-lactamases destroy the amide bond of a β-lactam in a two-step reaction. The positively charged residue of the enzyme attracts the negatively charged carboxylate group of the β-lactam antibiotic and binds with the help of hydrogen bonding. The β-lactam is acylated. A strategically positioned water molecule deacylates the β-lactam antibiotic and regenerates the enzyme β- lactamase[7].

3.8.1.1 Classification of β-lactamases[40]:

β-lactamases are classified according to two properties, the functional and molecular level.

3.8.1.1.1 Molecular classification:

The structural classification by Ambler includes four molecular classes:

A) The penicillinases and carbapenamases

B) The Metallo-Beta-Lactamases.

C) The Cephalosporinases.

D) The Oxacillinases and carbapenemases.

The enzymes in Classes A, C and D have serine in their active site. The MBLs bear zinc in their active site.

3.8.1.1.2 Bush Jacoby Classification:

They classified the beta lactamases into 4 groups (1-4) and 5 subgroups (a-f)

Group 1: cephalosporinases that resist to clavulanic acid, comparable with the class C in Ambler scheme.

Group 2: consists of penicillinases and cephalosporinases that are inhibited by clavulanic acid, comparable with the class A and D in Ambler classification.

Subgroup 2a: composed of penicillinases.

Subgroup 2b: consists of broad spectrum beta lactamases, inactivates both penicillins and cephalosporins. There are sub-subgroups, 2be and 2br.

Sub-subgroup 2be: Extended spectrum beta lactamases that can hydrolyse the third generation cephalosporins. It also hydrolyzes monobactams.

Sub-subgroup 2br: This group contains the enzymes that are inhibited by clavulanic acid and sulbactam. This is named 'r' due to its reduced ability to bind to clavulanic acid and sulbactam.

Subgroup c: This group have the property to inactivate carbenicillin more efficiently than benzyl penicillin.

Subgroup 2d: Enzymes in this group able to hydrolyze cloxacillin and carbenicillin more than the benzyl penicillin and also inhibited by clavulanic acid.

Subgroup 2f: This group consists of serine based carbapenemases but is different from zinc based carbapenemases in group 3.

Group 3: They are zinc based carbapenemases or metallobeta lactamases comparable with the Ambler class B. These enzymes hydrolyze penicillins, cephalosporins and carbapenems.

Group 4: Composed of penicillinases that resist to clavulanic acid and not comparable with any of the group in the Ambler class.

3.8.1.2 Extended Spectrum Beta Lactamase.

ESBLs are the enzymes that are capable of hydrolyzing penicillins, first, second and third generation cephalosporins and monobactum but do not hydrolyze cephamycins or carbapenems and are inhibited by β-lactamase inhibitor. They belongs to group 2be and group 2d of Bush- Jacoby- Medeiros Classification. ESBL is commonly acquired through large plasmids that holds many different resistant genes.

3.8.1.2.1 ESBL types

3.8.1.2.1.1 TEM

All the beta lactamase among gram negative bacteria known until 1965, were chromosomally mediated. The first plasmid mediated beta lactamase was first identified from an *E.coli* isolate from a patient named Temoneira from Greece, hence the name TEM[41]. They are the most common β-lactamase among gram negative bacteria. TEM is able to hydrolyze penicillins and narrow spectrum cephalosporins.

3.8.1.2.1.2 SHV

The more frequently encountered ESBL among clinical isolates are the SHV- type ESBL [42]. SHV type beta lactamases are often chromosomally encoded in *Klebsiella pneumoniae*, that are transferred to plasmids. In 1983, plasmid mediated β-lactamase which efficiently hydrolyzes cefotaxime was discovered in *klebsiella ozanae* isolate from Germany [43]. SHV-type β-lactamases are found primarily in *K. pneumoniae* strains[44,45].

3.8.1.2.1.3 CTX-M

This is non-TEM, non-SHV type of ESBL, reported in *E.coli* isolate from Munich in Germany[45]. The name CTX reflects its ability to hydrolyze cefotaxime. CTX-M type β-lactamases shows resistance to Cefotaxime but ceftazidime MIC are in the susceptible range[46]. However some CTX-M type ESBL also confers resistance to ceftazidime [46]. Karim et al in his study showed an outbreak of CTX-M type ESBL in New-Delhi[47].

3.8.1.2.1.4 OXA

OXA type β-lactamases has the ability to hydrolyze oxacillin, and hence the name OXA. The OXA type β-lactamases was first isolated from *pseudomonas areuginosa* isolates from Turkey. OXA-1 is the most common type in this group. OXA-1 has been found in about 1-10% of *E.coli*,isolates [48].

3.8.1.2.1.5 PER

PER was first described in *pseudomonas areuginosa*[49] . PER means *Pseudomonas* Extended Resistance. PER hydrolyzes penicillins and cephalosporins and are inhibited by clavulanic acid inhibitors. They are also detected in *salmonella spp, Acinetobacter spp,E.coli, Klebsiella pneumonia, proteus mirabilis*[50].

VEB, SFO-1, TLA-1,BEL-1 and there are other non-TEM, non-SHV ESBLs .

3.8.1.3 INDIAN SCENARIO:

Various studies from India quoted the prevalence of ESBL among Enterobactericeae varying from 20- 65%[51-53] (Vaidya et al., 2011; Kaur and Rudhresh et al.,2011).

Mohamudha parveen et al , in their study reported that about 60.8% of *Escherichia coli* was ESBL producer in a tertiary care hospital,pondicherry[54]in 2012. Patak et al 2012, from Ujjain in his study stated that the prevalence of ESBL producing *Escherichia coli* were about 69%[55]. The prevalence rate of ESBL in *Escherichia coli* isolates was 67% reported by Sarma et al from Guwahati[56].

There are some studies that showed higher prevalence rates in India. Chitnis et al 2011, revealed in his study from North India that 80.6% were extended spectrum beta - lactamase producers[57]. Priyadarshini et al 2011 from Salem reported 79.4% of the resistant isolates were ESBL producers[58].

3.8.1.4 GLOBAL SCENARIO:

Various studies across the world have shown different rates of ESBL production in *Escherichia coli* isolates. Mark E. Rupp *et al* observed that 10–40% of strains of

Escherichia coli and Klebsiella pneumoniae express ESBLs in many parts of the world[59]. The prevalence rate ranges from 28% - 52% in countries like Iran(Zahra Shahandesh et al, Iraj Sedighi et al)[60,61].

Prevalence of ESBL is very low in some countries when compare to India. . Mulvey et al in their study stated that 1.79% of the *Escherichia coli* were ESBL producers with SHV and CTX-M gene in Canada[62]. The author also quoted that lower prevalence of ESBLs was also prevalent in Netherland of about 1% .

3.8.2 QUINOLONE RESISTANCE

Bacterial resistance to quinolones may occur by several mechanisms[63]:

- chromosomal mutations in the structural genes *(gyrA, gyrB, parC,* and *parE)* that encodes the DNA gyrase and topoisomerase IV;
- Alteration in the outer membrane permeability to the drug;
- expression or overexpression of energy-dependent multidrug efflux pump AcrAB;
- Plasmid-mediated resistance genes *(qnrA, qnrB,* and *qnrS)* that prevents the binding of quinolones to DNA gyrase and topoisomerase IV; and
- Acquiring plasmid containing the resistance gene *aac(6)-Ib-cr,* which encodes a variant aminoglycoside acetyltransferase capable of modifying quinolones (selective only for ciprofloxacin and norfloxacin) and reducing their activity.

The gene qnr was found on plasmids varying in size 54 to 180kb inclinical isolates of *E.coli.* These confers low level resistance to quinolones.

3.8.2.1 Prevalence of quinolone resistance:

The prevalence of quinolone resistance among *Escherichia coli* was <3% in 2000 to 17.1% in 2010[34]. In India quinolone resistance in different studies varies from 38% to 90% (Gupta N *et al*, Kothari *et al*, Varughese et al).

3.8.3 Resistance Mechanism of Aminoglycosides, macrolides and other antibiotics:

There are various mechanisms responsible for the intrinsic resistance of the organism towards many antibiotics. The loss of the OprD porin that leads to resistance of antibiotics, such as aminoglycosides, and colistin. The Aminoglycoside-modifying enzymes often encoded on transposons and integrons result in various combinations of resistance to gentamicin, tobramycin or amikacin. These enzymes also carry resistance determinants for other classes of antibiotics such as sulfonamides, β-lactams and chloramphenicol. Along with the impermeability mutations these Aminoglycoside-modifying enzymes result in the broad-spectrum aminoglycoside resistance. It has been described that the broad-spectrum aminoglycoside resistance is due to a gene rmtA.

The intrinsic mechanism which includes efflux pump systems mediated by some regulatory genes can cause expulsion of β -lactams, fluoroquinolones, macrolides, sulfonamides, chloramphenicol, tetracycline, and trimethoprim.

The overproduction of the efflux pump is due to the upregulation of the mexR gene which can cause resistance to antibiotics such as quinolones, penicillins, cephalosporins and aztreonam.

3.9 PHENOTYPIC METHODS FOR ESBL DETECTION :

Extended spectrum beta lactamases (ESBLs) produced by *E.coli* possess a serious problem due to their ability of transmission between the same as well as different bacterial species. There are many different methods to detect ESBLs which may be performed in the laboratory.

3.9.1 ESBL screening tests:

The current Clinical Laboratory Standard Institute guidelines[67] for detection of ESBL in *E.coli* includes an initial screening test with any of the two following drug such as cefotaxime, ceftazidime, ceftriaxone, cefpodoxime and aztreonam . The use of more than one of the drug improves the sensitivity of the test. Cefpodoxime and ceftazidime shows increased sensitivity for ESBL detection.

3.9.1.1 Disc Diffusion method:

According to CLSI guidelines, isolates showing zone of inhibition of ≤17 mm for cefpodoxime 30µg, ≤22 mm for ceftazidime 30 µg, ≤25 mm for ceftriaxone 30µg and ≤ 27mm for Cefotaxime 30µg and aztreonam 30µg are considered to be potential ESBL producers.

3.9.1.2 Broth dilution method.

Mueller-Hinton(MH) broth media containing dilutons of Cefotaxime, Ceftazidime, Cefepime, Aztreonam in doubling dilution with concentration ranging from 1µg/ml are prepared . Bacterial suspension of 0.5 macfarland standard is inoculated in each well according to CLSI guidelines. The plates are incubated aerobically at 37ºC overnight. MIC ≥2µg/ml may indicate ESBL production. The organisms that are positive in screening test for ESBL are further confirmed with phenotypic confirmatory test[7]. For cefpodoxime MIC ≥8µg/ml indicate ESBL production[7].

3.9.2 Phenotypic confirmatory test

3.9.2.1 Combined Disk Diffusion Test (CDDT):

The test strain with 0.5 macfarland standard is inoculated on to the Mueller Hinton agar plate. A Cefotaxime (30 µg) disc and a combination disc containing Cefotaxime 30 µg and clavulanic acid 10 µg is placed on the inoculated plate. Similarly ceftazidime 30µg and ceftazidime 30µg–clavulanic acid 10µg combination disc are used.The plate is incubated aerobically at 35°C for 16 to 18 hours. A ≥ 5mm increase in a zone diameter of Cefotaxime-Clavulanate vs Cefotaxime alone confirms the ESBL production.

Mohammed Hisam et al in a Comparative analysis of detection methods of ESBL among *Enterobacteraceae* found 100% sensitivity for CDDT & 66.6 % sensitivity for DDST[69] .

Yves De Gheldre et al reported that the sensitivity of CDDT in *E.coli* with Ceftazdime with Clavulanic acid and Cefotaxime with Clavulanic acid was 89% and 84% respectively and using both the drug has sensitivity of 100%, also showed that the specificity of CDDT in Escherichia coli with Ceftazdime with Clavulanic acid and Cefotaxime with Clavulanic acid was 88%[70].

3.9.2.2 Double Disk Synergy Test (DDST):

About 0.5 macfarland standard of the test strain is inoculated on to the Mueller Hinton agar plate. A cefotaxime 30µg disc and a ceftazidime 30 µg disc was placed 20 mm centre to centre from a combination disc containing Amoxicillin 30 µg with Clavulanic acid 10 µg. The enhanced zone of inhibition in the area between combination disc and the cefotaxime disc and Ceftazidime is interpreted as a positive.

HO PL et al reported a sensitivity of 83.8% for DDST at a single interdisc width of 30mm owed increased sensitivity of 97.9% by decreasing the interdisc width to 20 mm. sensitivities of Ceftazidime, Ceftriaxone and Cefpodoxime are 57.7%,98.6% and 99.3% respectively[71].

3.9.2.3 Broth dilution test:

ESBL can also be confirmed phenotypically by broth microdilution assays using cepalosporins (ceftazidime, Cefotaxime) at a concentration of 0.25/4 to 128/4 µg/ml of clavulanate (4µg/ml). A two to three fold reduction in the MIC of well containing cephalosporin in the presence of clavulanate when compared to cephalosporin alone is considered to be ESBL production.

3.9.2.4 ESBL E-test (Epsilometer test):

The ESBL E-Test is a drug-impregnated strip, one end of the strip has a stable concentration gradient of cephalosporin and the other end has a gradient of cephalosporin plus clavulanate in a constant concentration. Ratio of ceftazidime MIC and ceftazidime-clavulanic acid MIC equal to or greater than 8 or deformation of ellipse or phantom zone is considered to be positive for ESBL production. These strips can be used for both screening and confirmatory test for ESBL detection.

The sensitivity of the E-test in various studies for ESBL detection is between 87-100% and sensitivity is 95-100%[7].

3.9.2.5 Vitek ESBL

The Vitek ESBL test utilizes ceftazidime (0.5µg/ml) discs alone and these discs combined with clavulanic acid (0.4 µg/ml). Once the growth control well reached a set threshold, automated analysis of the wells are performed. A reduction in the growth of cephalosporin wells containing clavulanic acid, compared with the level of growth in the wells with the cephalosporin alone, indicates a positive result[7].

3.10 PHENOTYPIC DETECTION OF QUINOLONE RESISTANCE:

3.10.1 Disc Diffusion Method:

Bacterial suspension of 0.5 mac farland standard is prepared. The bacterial suspension is inoculated in the Muller-Hinton agar plate as for routine disc diffusion procedure. Ciprofloxacin 5µg disc and Norfloxacin10 µg disc are placed on the plate. The plate is incubated aerobically at 35°C for 16 to 18 hours. The zone of inhibition of the discs are measured. If the zone of inhibition is ≤ 15mm for Ciprofloxacin and ≤ 12mm for Norfloxacin may indicate quinolone resistance.

3.10.2 Quinolone resistance by Microbroth Dilution Method

Mueller-Hinton broth media containing serial two fold dilution containing Ciprofloxacin with concentration ranging from 0.25 to 512 µg/ml are prepared and is placed in 96 well microtitre plate. Bacterial suspension of 0.5 macfarland standard is prepared from 4-5 colonies from 24 hour culture and is inoculated in each well according to CLSI guidelines. The plates are incubated aerobically at 37ºC overnight. MIC of ≥4µg/ml indicate quinolone resistance.

3.10.3 Quinolone resistance by E-test:

The ciprofloxacin E-Test strip with dilution range of Ciprofloxacin (0.002 to 32µg/mL) was used. The E-test are done according to manufacturer's instructions. MIC ratio of Ciprofloxacin ≥4µg is considered to be positive for Quinolone resistance.

3.11 Coexistence Of Quinolone Resistance Among Esbl *E.Coli*:

The ESBL producing bacteria are often assosiated with multi drug resistance, as the genes responsible for the other resistance mechanism exist on the plasmid carrying the ESBL gene. Quinolone resistances among extended-spectrum beta-lactamase (ESBL) production are common in Enterobacteriaceae[72].

Frank et al, 2006, reported that among 17 ESBL producing *E.coli* 52.94% (n=9) were resistance to quinolone in Central African Republic[73].

Pakzad I et al, 2011 in his study reported that about 64.2% of ESBL producing *Escherichia coli* was resistance to quinolones in Iran[74].

In studies conducted by Lautenbach et al in USA (2001) concluded that of the 77 ESBL-*Escherichia coli and Klebsiella* infections, 43 (55.8%) were fluoroquinolones resistant[75].

3.11.1 Genotypic detection of plasmid mediated quinolone resistance

Although phenotypic methods are cost effective and easy to use, there is possibility of false positive and false negative results in phenotypic methods. Genotypic methods are the standard methods for identification of the quinolone resistance genes are available. There are various genes responsible for the plasmid mediated quinolone resistance such as *qnrA, qnrB, qnrD, qnrS, aac-(6')-Ib-cr*. Among these *qnrA, qnrB* and *aac-(6')-Ib-cr* are the more prevalent worldwide[76,77,78].

4. MATERIALS AND METHOD

In the present study 100 consecutive non-duplicate isolates of *Escherichia coli* obtained from various clinical specimens like pus, urine, wound swab, sputum, blood, pleural fluid and CSF from inpatients admitted in Tirunelveli medical college, Tirunelveli were included. The study period was from April 2015 to May 2016.

4.1 METHODOLOGY

4.1.1 Collection and Processing Of Various Samples:

All samples were collected under aseptic precautions by standard procedures and processed according to standard guidelines. Brain heart infusion broth was used for the blood culture. The broth which showed turbidity was sub-cultured onto the MacConkey Agar and blood agar media using sterile technique.

The urine specimens were centrifuged and inoculated on to the culture Nutrient agar and MacConkey Agar media. The wound swab and the other specimens such as pus were inoculated in the appropriate culture media and was also used for direct gram stain. The incubation was done at 37˚C for a period of 18-24 hours aerobically.

Among the 1247 samples processed ,100 non replicate isolates of *Escherichia coli* from clinical samples were taken in the study.

4.1.2 Ethical clearance and Informed consent :

The ethical committee clearance was obtained from our institution and informed consent was obtained from all patients included in the study.

4.1.3 Proforma:

The proforma was filled with the details like name, age, sex, ward, clinical diagnosis, risk factors, undergone any surgery, duration of hospital stay and other parameters significant to the present study.

4.1.4 Sample storage:

The *Escherichia coli* isolates were sub-cultured on to nutrient agar slope and stored at 2 to 8˚C. The isolates were sub-cultured in every two weeks.

4.1.5 Identification of *Escherichia coli*:

The *Escherichia coli* isolates were identified by gram staining, cultural characteristics and standard biochemical reactions.

4.1.6 ANTIBIOTIC SUSCEPTIBILITY TESTING:

Antibiotic susceptibility testing was done for all isolates of *Escherichia coli* by Kirby Bauer disc diffusion method according to the recommendation of the clinical and laboratory standards institute (CLSI-2016).

4.1.6.1 Kirby-Bauer's Disc Diffusion (DD) method:

About 3-5 colonies of the test organism were inoculated in 2 ml of peptone water and incubated for 2-4 hrs at 37˚C. The inoculum suspension was compared with 0.5 McFarland (1.5×10^8 CFU/ ml) standard suspension by positioning the tube side by side against a white card containing several horizontal black lines. The turbidities were compared by looking at the black lines through the suspensions. Once standardized, the inoculum suspension was used within 15 minutes of preparation.

4.1.6.1.1 Procedure.

- A sterile cotton swab was soaked in the inoculum and a lawn culture was made on to the Muller-Hinton agar (MHA).
- By rotating the swab against the inner side of the test tube, excess broth was expressed.
- Swab was streaked evenly over the surface of the medium in three directions; the plate was rotated approximately 60° for even distribution.
- The surface of the agar plate was allowed to dry for 3-5 minutes.
- The discs were placed about 15mm from the edge of the plate and not closer than about 25mm from disc to disc with the help of a sterile needle.
- Only six discs were applied on a 90mm plate. Each disc was lightly pressed down to ensure its contact with the agar.
- The plate was inverted and incubated at 35˚C aerobically for 18-24 hours.

The zone size was recorded and interpreted according to the CLSI guidelines 2016.

4.1.7 ESBL detection by phenotypic methods:

4.1.7.1 Initial screening by disc diffusion method:

All the isolates of *Escherichia coli* are tested for the susceptibility for Ceftazidime 30 µg, Cefotaxime 30 µg and Ceftriaxone 30 µg by disc diffusion method as per the CLSI guidelines. According to the CLSI guidelines the zone of inhibition was measured and interpreted. Zone size of ≤ 22mm for ceftazidime, ≤ 25mm for ceftriaxone and ≤

27mm for Cefotaxime were taken as resistant. Strains that were resistant to any of the three cephalosporins tested were considered as potential ESBL producer.

4.1.7.2 Detection of ESBL:

The isolates which were found to be resistant to any one of the three drugs were selected and subjected to various phenotypic methods like CDT, DDST, and ESBL E Test to detect ESBL in test organism. ATCC 25922 *Escherichia coli* were used.

4.1.7.2.1 Combined Disc Diffusion Test (CDDT):

As per the CLSI guidelines, the 24 hour young culture isolate of the test strain is inoculated on to the Muller Hinton agar plate. Ceftazidime disc 30 µg, Cefotaxime 30µg, Ceftazidime with Clavulanic Acid (30µg/10µg) disc and Cefotaxime with Clavulanic acid(30µg/10µg) disc were placed on the dried agar plate. The plate was incubated at 37˚C aerobically for 16-18 hours. The zone of inhibition was measured .The increase in the diameter of the zone by more than or equal to 5mm (≥5mm) for the Ceftazidime-Clavulanic acid and Cefotaxime-clavulanic acid combined disc when compared to Ceftazidime and Cefotaxime alone respectively was considered ESBL positive.

4.1.7.2.2 Double Disc Synergy Test (DDST):

The test strain was inoculated as lawn culture on to the Muller Hinton agar plate as per the CLSI guidelines. .Amoxycillin-clavulanic acid(30/10µg) disc was placed in the middle of the plate. Discs of ceftazidime (30 µg), cefotaxime (30µg) were placed at a distance of 20 mm from center to center to the amoxicillin-clavulanic acid combination disc in a straight line. The plates were incubated at 37ºC for 16-18 hours, aerobically overnight. Isolates which showed an enhanced of the zone of inhibition towards the amoxicillin-clavulanic acid side than on the side without amoxicillin-clavulanic acid, were confirmed as ESBL producers.

4.1.7.2.3 ESBL E Test:

ESBL E test strip is a unique strip coated with mixture of Cefotaxime + Clavulanic acid and Cefotaxime in a concentration gradient manner. The strip is made of porous material and the antibiotics are distributed evenly on either side of the strip. It was used to determine the minimum inhibitory concentrations of the drug for the test strain. E test ESBL strip has a double sided antibiotic concentration in a range of Cefotaxime(CTX)-0.25-16 µg/ml and Cefotaxime-Clavulanic acid (CTX+)-0.016-1µg/ml with a fixed concentration of Clavulanic acid 4µg/ml.

Procedure:

The inoculum was prepared with 4-5 colonies from 24 hour young culture and inoculated as a lawn culture on to the Muller Hinton agar after adjusting the turbidity to 0.5 Mc Farland standard. The strip was then taken with a sterile forceps or E-test applicator and applied to the dried agar surface with the MIC scale facing upwards. The plate was incubated aerobically for 16-18 hourrs at 37^0C.

Interpretation:

The plate was read after the determined time of incubation. The MIC of the isolate was read where the zone of inhibition intersects the strip. The MIC for CTX/CTX-clavulanic acid ≥ 8 or deformation of ellipse or phantom zone was considered positive for ESBL production.

4.1.8 Quinolone resistance detection by phenotypic methods:

4.1.8.1 Quinolone resistance detection by E-Test:

The strains that were resistant and intermediate sensitive in disc diffusion test with ciprofloxacin and norfloxacin were subjected to Ciprofloxacin E-test.

The inoculum was prepared with 4-5 colonies from 24 hour young culture and inoculated as a lawn culture on to the Muller Hinton agar after adjusting the turbidity to 0.5 Mc Farland standards. The Ciprofloxacin E- test strip container was taken from the freezer and kept at room temperature for 15 minutes before opening. The strip was then taken with a sterile forceps or E test applicator and applied to the dried agar surface with the MIC scale facing upwards. The plate was incubated aerobically for 16-18 hrs at 37^0C.

The plate was read after the determined time of incubation. The MIC of the isolate was read where the zone of inhibition intersects the strip. The MIC of > 4µg for Ciprofloxacin or deformation of ellipse or phantom zone was considered positive for ciprofloxacin resistance.

4.1.9 Plasmid mediated quinolone resistance detection by PCR:

The quinolone resistant among the ESBL producing *E.coli* isolates were further tested for the presence of plasmid mediated *qnrA*, *qnrB* and *aac-6-Ibr* gene by Real-Time PCR. The PCR kit was procured from Helini Biomolecules, Chennai, India. According to the manufacturer's instructions, the procedure was performed.

Requirements:

- Micro Pipettes variable volume 0.5-10 µl, 10-100 µl, and 100-1000 µl
- Sterile tips.

- Vortex mixer
- Water bath
- 13,000 rpm Centrifuge (Refrigerated) with rotor for 1.5ml reaction tubes
- 1.5ml/2ml centrifuge tubes
- Thermo cycler (Biorad CFX 96)
- Computer for data storage

4.1.9.1.DNA Extraction:

The DNA extraction procedure yields purified DNA of more than 30kb in size obtained after the lysis of the cell.

4.1.9.1.1Components of extraction

- Phosphate buffered saline
- Lysozyme
- Digestion buffer
- Binding buffer
- Proteinase K
- Internal control template
- Isopropanol
- 70% Ethanol
- Elution buffer

4.1.9.1.2 Storage and stability:

The bacterial genomic DNA extraction kit was stored at room temperature.

The proteinase K and Lysozyme were stored at -20 ^{0} C.

4.1.9.1.3 Bacterial pellet preparation:

About 4-6 colonies of the test strains were inoculated in peptone water and incubated at 37^{0}C overnight. 1-1.5 ml of bacterial culture was transferred into the sterile 2ml centrifuge tube. The tube was centrifuged at 8000rpm for 5 minutes at room temperature. The supernatant was discarded and the pellet was used for the DNA extraction.

4.1.9.1.4. Principle:

The cells are lysed with the enzyme Proteinase K and the nucleases are inactivated by chaotropic salt. The nucleic acids are bound to special silica fibres in the spin column tube. The cellular components contaminating the bound nucleic acid is removed in a series of rapid “wash and spin” steps. The elution releases the nucleic acids from the silica fibre.

4.1.9.1.5 DNA extraction :

The bacterial pellet was suspended in 200 µl phosphate buffer saline and 180 µl of digestion buffer and 20 µl of lysozyme were added and vortexed for 10 seconds. The mixture is incubated and 200 µl of binding buffer, 20 µl of proteinase K and 5 µl of internal control template was added and mixed well by pulse vortex. It was incubated and then 300 µl of Isopropanol is added and mixed well. The entire sample was pipetted into the spin column. At 12,000rpm it was centrifuged for 1 minute. The flow through was discarded. The spin column was placed into the collection tube. 500 µl of 7% ethanol is added to the spin column. At 12,000rpm it was centrifuged for 1 minute. 75 µl of the pre-warmed (warmed at $56^{0}C$) Elution buffer was added to the centre of the spin column and incubated for 2 minutes at room temperature. It was centrifuged for 1 minute at 13,000rpm.The spin column was discarded and eluted DNA was taken for the PCR procedure.

4..1.9.2 PCR Amplification:

qnrA, qnrB **and** ***aac-(6')-Ib-cr*** **primer & probe mix:**

The *qnrA, qnrB* and *aac-6'-Ib-cr* primer & probe mix consists of TaqMan probe which is florescent labeled with FAM, forward primer and reverse primer.

For *qnrA* gene:

Forward primer: CACTTCAGCTATGCCGATCT

Reverse primer: TCGCATTCCCTGAACTCTATG

Probe sequence: TGCCGTCTGTCTTTGGCTAACTTCA

For *qnrB* gene:

Forward Primer: ACCAATCTAAGCTACGCCA

Reverse Primer: GGAGAGATCTGAACCACTGAAC

Probe: CGTTGGATAGGTGCCCAGGTACTG

For *aac-(6')-Ib-cr* gene:

Forward Primer: ACGGGTCCGTTTGGATCTTG

Reverse Primer: ACTGGGCAAAGGCTTGGGAA

Probe sequence: TGACCTCGGGATCATTGAACAGCA

4.1.9.2.1 Internal Control primer & probe Mix

The internal control primer & probe mix consists of TaqMan probe which is florescent labeled with HEX, forward primer and reverse primer. The internal control is included to make sure that PCR inhibitors are removed during the DNA extraction procedure and the performance of PCR mix ingredients are good. The internal control if amplified shows that PCR inhibitors are not present in the sample and the nucleic acid purification is optimum. It helps to rule out false negative results.

4.1.9.2. 2Positive control:

qnrA positive template *qnrB* and *aac-(6')-Ib-cr* positive templates are used as positive control. A positive result indicates that the primers and probes worked correctly in that particular experiment.

4.1.9.2.3 Negative control:

Nuclease free water as used as a negative control. A negative result indicates that there is no contamination in the reagents.

4.1.9.2.4 Materials required:

- Real time PCR machine with FAM/JOE channel
- 0.2ml PCR tubes/8well strips
- Micropipette and tips

4.1.9.2.5 PCR detection mix:

The *qnr*A, *qnr*B and and *aac-(6')-Ib-cr* reaction mix for the samples consisted of probe PCR master mix 10µl, *qnr*A primer probe mix 10µl, *qnr*B and internal control primer probe mix 10µl, purified DNA sample 5µl and a total volume of 25µl.

For positive control mix, 5µl of positive control template was added instead of sample DNA and for negative control mix, 5µl of nuclease free water was added instead of sample DNA.

The negative control is added first followed by samples and finally positive control was added to prevent cross contamination.

The PCR detection mix was centrifuged after adding all the ingredients and they were placed in the thermo cycler where the PCR reaction was allowed to occur.

Table:1. *qnr*A reaction mix for samples

S.No	**Components**	**Volume**
1.	Probe PCR Master Mix	10µl
2.	*qnr*A Primer Probe Mix	5µl
3.	Purified DNA sample	10µl
4.	Total reaction volume	25µl

Table:2. *qnr*B reaction mix for samples

S.No	**Components**	**Volume**
1.	Probe PCR Master Mix	10µl
2.	*qnr*B primer probe mix	5µl
3.	Purified DNA sample	10µl
4.	Total reaction volume	25µl

Table:3. *aac-(6')-Ib-cr* reaction mix for samples

S.No	Components	Volume
1.	Probe PCR Master Mix	10µl
2.	*aac-(6')-Ib-cr* Primer Probe Mix	2.5µl
3.	Ic primer probe mix	2.5 µl
3.	Purified DNA sample	10µl
4.	Total reaction volume	25µl

4.1.9.3 PCR amplification steps:

4.1.9.3.1 Initial Denaturation:

This is the first step in the amplification procedure. The thermocycler raises the temperature to 95˚C for fifteen minutes for Taq enzyme activation.

The temperature is elevated to 95˚C for 20 seconds. The double stranded template DNA gets separated into two complementary strands.

4.1.9.3.2 Annealing:

When the temperature is decreased to 55˚C for 20 seconds the complementary binding of the two specific oligonucleotide primers to the DNA template take place.

4.1.9.3.3 Extension:

The DNA polymerase extends the primers when the temperature is increased to 72˚C for 20 seconds. The template DNA is synthesised using deoxynucleotides (dNTPs) in the reaction mixture. Two single stranded DNA templates and newly synthesized complementary DNA strands attach together to form new double stranded DNA copies.

Every copy of newly formed DNA may function as a template for further amplification. The products will be amplified in an exponential manner in each cycle. At the end of 40 cycles, the final PCR products will have 2n copies of template DNA. Data analysis was made at the end of extension and the computer produces the cross threshold (Ct) value by calculating the fluorescence emitted at the end of each cycle.

Table:4. Amplification profile for *qnr*A, *qnr*B and *aac-(6')Ib-cr* gene:

`	**Step**	**Time**	**Temp**
	Taq enzyme activation	15min	95^0 C
45cycles	Denaturation	20sec	95^0 C
	Annealing/ Data collection	20sec	55^0 C
	Extension	20sec	72^0 C

*qnr*A, *qnr*B, *aac-(6')-Ib-cr* =FAM channel

Internal control = HEX channel

4.1.9.4 Interpretation of the results:

4.1.9.4.1 Negative control:

The negative control reactions should not exhibit fluorescence growth curves that cross the threshold line.

4.1.9.4.2 Positive control:

Positive control reactions should produce positive results before 36 cycles.

4.1.9.4.3Test sample positive:

When all the controls fulfill the quantified requirements, a test sample is measured positive. Any samples which exhibit fluorescence that cross the threshold line at or after 38 cycles should be retested.

4.1.9.4.4 Test sample negative:

When all controls fulfill the quantified requirements, a test sample is measured negative.

4.1.9.4.5 Internal control interpretation:

According to the protocols (assuming 100% extraction efficiency) the CT value is expected within 21-31 for the internal control. This may differ depending on the extraction efficiency, the quantity of elute added to the PCR reaction and the settings of machine. If the amplifying sample had high genome copy number then the internal control may not produce an amplification plot. This can be interpreted as positive result rather invalidates it.

Table:5. Interpretation of results

Test Sample	**Negative control**	**Internal control**	**Positive control**	**Interpretation**
Positive	Negative	Positive	Positive	**Positive**
Negative	Negative	Positive	Positive	**Negative**
Negative	Negative	Negative	Negative	**Repeat**
Positive	Positive	Positive	Positive	**Repeat**

5. RESULTS

5.1 Study Description:

The study was conducted at the Department of Microbiology, Tirunelveli Medical College, over a period of one year from May 2015 to June 2016.A total of 100 *Escherichia coli* isolates from various clinical samples were included in the study. These isolates were subjected for ESBL screening and quinolone resistance by disc diffusion test. The resistant isolates were confirmed for ESBL with phenotypic tests like CDDT (Combined Disc Diffusion Test), DDST (Double Disc Synergy test). Quinolone resistance were confirmed by E test. Coresistance of quinolone resistance among ESBL *E.coli* were identified. The isolates with ESBL and quinolone resistance were tested for the presence of plasmid mediated quinolone resistance gene by Real-Time PCR. The isolates were tested for the sensitivity patterns to the antibiotics and the other associated risk factors were analyzed.

5.2 Statistical Analysis:

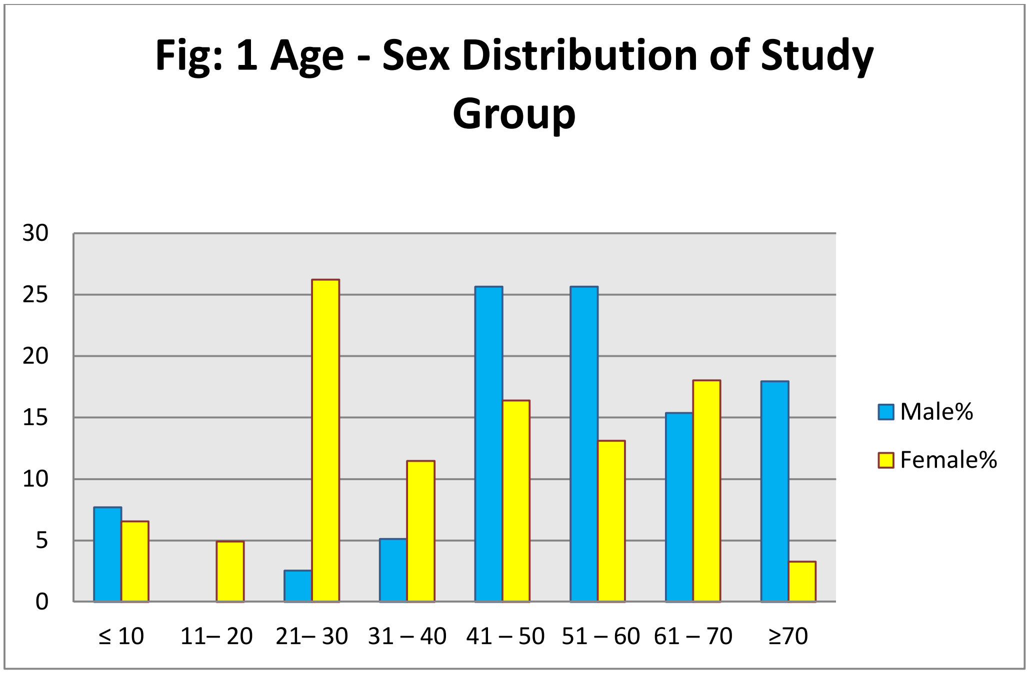
Data regarding the subjects were defined in terms of percentages. The statistical measures were completed with the help of the statistical software IBM SPSS statistics 20. The p values < 0.05 was considered as significant ($p < 0.05$) in Chi square and McNemer test.

5.3 Age – Sex distribution of the *E.coli isolates*

Table 6   Age and gender distribution of the *E.coli* isolates

Age (years)	Male		Female		Total	
	No	%	No	%	No	%
≤ 10	3	7.69	4	6.56	7	7
11– 20	-	-	3	4.92	3	3
21– 30	1	2.56	16	26.23	17	17
31 – 40	2	5.13	7	11.47	9	9
41 – 50	10	25.64	10	16.39	20	20
51 – 60	10	25.64	8	13.11	18	18
61 – 70	6	15.38	11	18.03	17	17
≥70	7	17.95	2	3.28	9	9
Total	39	99.99	61	99.99	100	100

Out of 100 isolates, 64 isolates were between the age group of 41 and >70 years. Among the 100 isolates *E.coli* were isolated more from females(61%) than males(39%). The mean age of male was 53.40years and that of female was 42.47 years.(Table 6, Fig1)



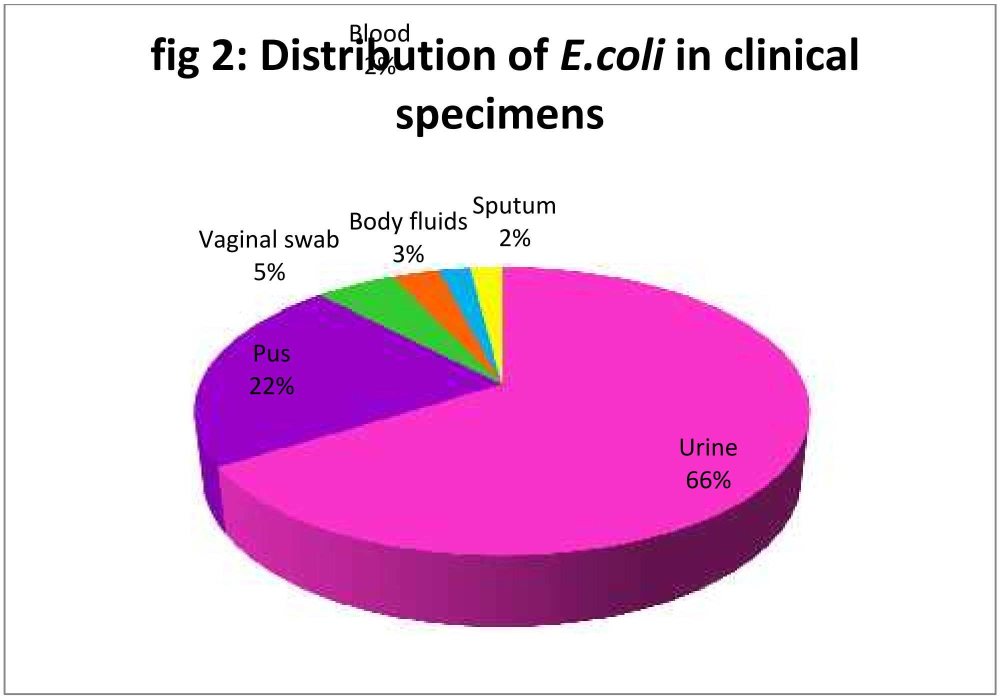
5.4 Distribution of *Escherichia coli* in clinical specimens:

The specimen wise distribution of *Escherichia coli* was analyzed and about 66 isolates were from urine samples. 22 and 5 samples were from pus and vaginal swab respectively. Two isolate were obtained from blood and sputum. Three isolates were from other body fluids (CSF and Ascitic fluid).(Table 7, Fig:.2.)

Table 7. Distribution of *Escherichia coli* in clinical specimens:

S.No	Specimen	Number	Percentage (%)
1	Urine	66	66
2	Pus	22	22
3	Vaginal swab	5	5
4	Body fluids	3	3
5	Blood	2	2
6	Sputum	2	2

Body fluids: CSF,Ascitic fluid



5.5 Ward wise distribution of *Escherichia coli* isolates:

Table 8: Distribution of *Escherichia coli* isolates in wards

Ward	No	%
Surgery	19	19
Medicine	20	20
Urology	27	27
O&G	23	23
IMCU	4	4
Nephrology	2	2
Paediatrics	4	4
Neuro surgery	1	1
Total	**100**	**100**

The above table 8 shows the distribution of *Escherichia coli* isolates among samples from various departments of the hospital. Urology department accounted for the majority of the *Escherichia coli* isolates 27 (27%) of the 100 isolates. 23% of isolates were from O&G department. 20% and 19% of isolates were from medicine and surgery department respectively. 4% isolates were from paediatrics and IMCU. 2% isolates were from nephrology department and 1% from neuro surgery department.

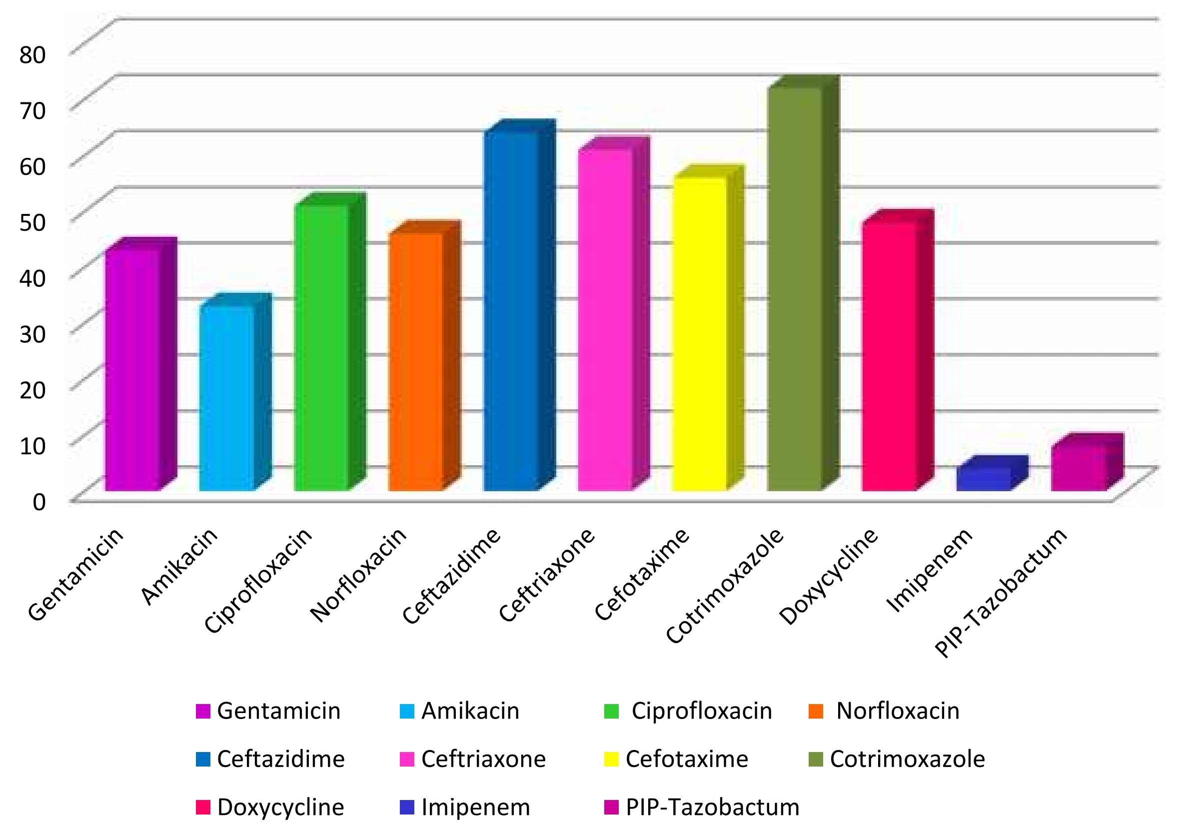
5.6 Antibiotic resistance patterns of *Escherichia coli* :

Table 9. Resistance patterns of *E.coli* to Antibiotics

Antibiotic discs	**No .of resistant Isolates**	**Percentage (%)**
Gentamicin (GEN) 10 µg	43	43
Amikacin (AK)30 µg	33	33
Ciprofloxacin (CIP) 5 µg	49	49
Norfloxacin (N) 10 µg	46	46
Ceftazidime (CAZ)30 µg	64	64
Ceftriaxone (CTR) 30 µg	61	61
Cefotaxime (CTX) 30 µg	56	56
Trimethoprim/sulfamethoxazole (COT)1.25/23.75µg	72	72
Doxycycline30 µg	48	48
Imipenem 10µg	4	4
Piperacillin - Tazobactam (100/10µg)	8	8

The overall resistance pattern of *E.coli* were shown in table 9. The highest resistance was shown to cotrimoxazole of 72%. Lowest resistance was with imipenem 4%. Among the cephalosporins, ceftazidime showed highest resistance of 64% followed by ceftriaxone and Cefotaxime with 61% and 56% respectively. Among the quinolones, ciprofloxacin showed more resistance (49%) than the norfloxacin (46%). The isolates were resistance to other antibiotics like gentamicin (43%), amikacin (33%), and doxycycline (48%). (Fig 3). Urine samples were also tested with nitrofurantoin which showed 16.67% of resistance(11/66).

fig 3. AntibioticResistance patterns of *E.coli*



5.7 Screening test for ESBL detection:

Tabl 10 screening for ESBLin *Escherichia coli* isolates by Disc Diffusion test:

Disc	ESBL producer	
	No	%
Cefotaxime 30µg	55	78.57
Ceftriaxone 30µg	59	84.29
Ceftazidime 30µg	63	90

The above Table.10 shows the results of initial screening test in the detection of ESBL determined by disc diffusion test using the discs, Cefotaxime (30µg), ceftriaxone(30µg) and ceftazidime(30µg). The zone of inhibition ≤27mm, ≤25mm, ≤22mm for Cefotaxime, ceftriaxone and ceftazidime respectively were taken as resistant. Isolates that were resistant to any of the cephalosporins tested were considered as potential ESBL producers. Among the100 *Escherichia coli* isolates 70 isolates showed resistance to third generation cephalosporins.

5.8 Analysis of various phenotypic methods for the detection of the ESBL production:

The 70 *Escherichia coli* isolates which showed resistance to third generation cephalosporins were evaluated by various phenotypic methods like CDDT, DDST and ESBL E test. Positive result with any of the three method is confirmed as ESBL producers. Out of the 70 screening positive isolates a totalof 63 isolates showed ESBL production. Among them 60 were confirmed by CDDT and 54 were positive with the DDST and 61 were positive with E test.(Table 11, Fig.4)

Table 11. Detection of ESBL by various phenotypic methods

Screening positive=70

Method	ESBL producer	
	No	%
CDDT	60	95.23%
DDST	54	85.71%
E-test	61	96.83%

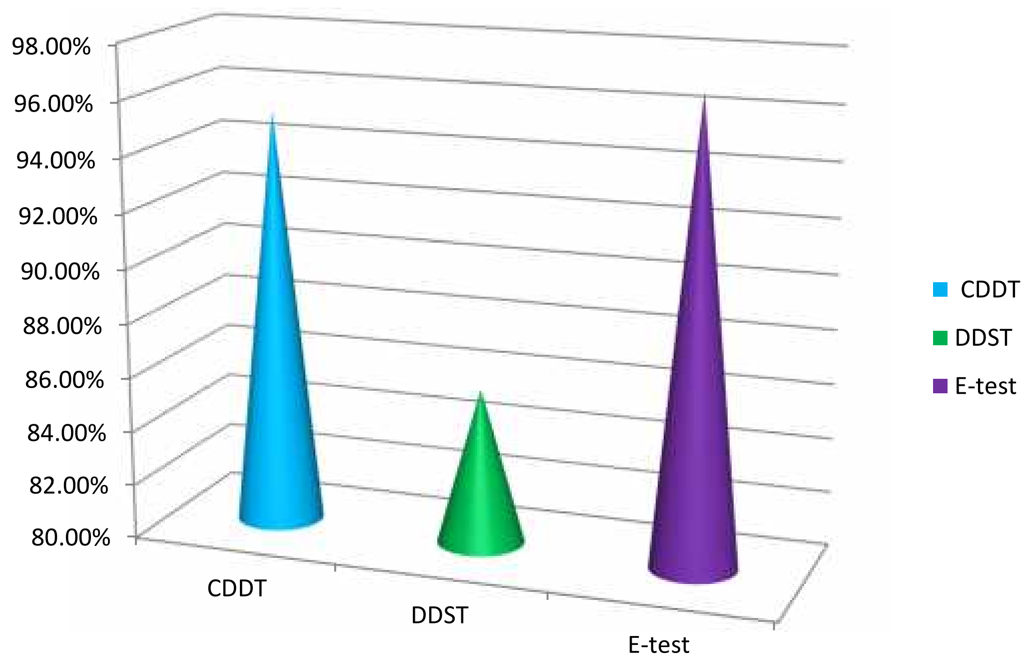


Fig. 4 Comparison of various phenotypic methods in ESBL detection

5.9 Comparison of CDDT and DDST for detection of ESBL:

The comparison of ESBL detection using CDDT and DDST was depicted in the table no 12 . Out of 70 isolates which were positive in screening test 60 were detected by CDDT and 54 were detected by DDST. Out of 70 screening positive isolates 10 were negative by CDDT and 16 were negative with DDST. Sensitivity and specificity of the DDST were 84% and 90% respectively. positive predictive value of the test is about 98% and negative predictive value is about 56.2%.

Table 12.Comparison of CDDT and DDST for detection of ESBL

Screening positive N=70

DDST	CDDT		TOTAL
	Positive	**Negative**	
Positive	53	1	**54**
Negative	7	9	**16**
	60	**10**	**70**

5.10 Comparison of CDDT and E-testT for detection of ESBL:

Table 13. Comparison of CDDT and E-test for detection of ESBL

E-test	CDDT		TOTAL
	Positive	**Negative**	
Positive	58	3	**61**
Negative	2	7	**9**
	60	**10**	**70**

Among 70 ESBL screening isolates, 61 isolates were positive with the E test. Three isolates with E test positive were negative with the CDDT and 2 isolate with E test negative were CDDT positive.

The sensitivity, specificity, positive predictive value and negative predictive value of ESBL E test were 97%, 70%, 95% and 78% respectively. (Table: 13). The kappa value denoting the measure of agreement is high (0.69).

5.11 Age-Sex-wise distribution of ESBL *Escherichia coli*

The table (14) shows age sex distribution of ESBL *E.coli* among the study group. ESBL were more in female than in male with a male female ratio of 1:1.1. ESBL were more common in the age group of >45 years to 60 years(42.86%).

Table 14. Age-Sex-wise distribution of ESBL *Escherichia coli*

AGE	MALE		FEMALE		TOTAL	
	NO	%	NO	%	NO	%
<15	1	3.33	2	6.06	3	4.76
16-30	-		2	6..06	2	3.17
31-45	5	16.67	1	3.03	6	9.52
46-60	11	36.67	16	48.48	27	42.86
>60	13	43.33	12	36.36	25	39.68
	30	100	33	99.99	63	99.99

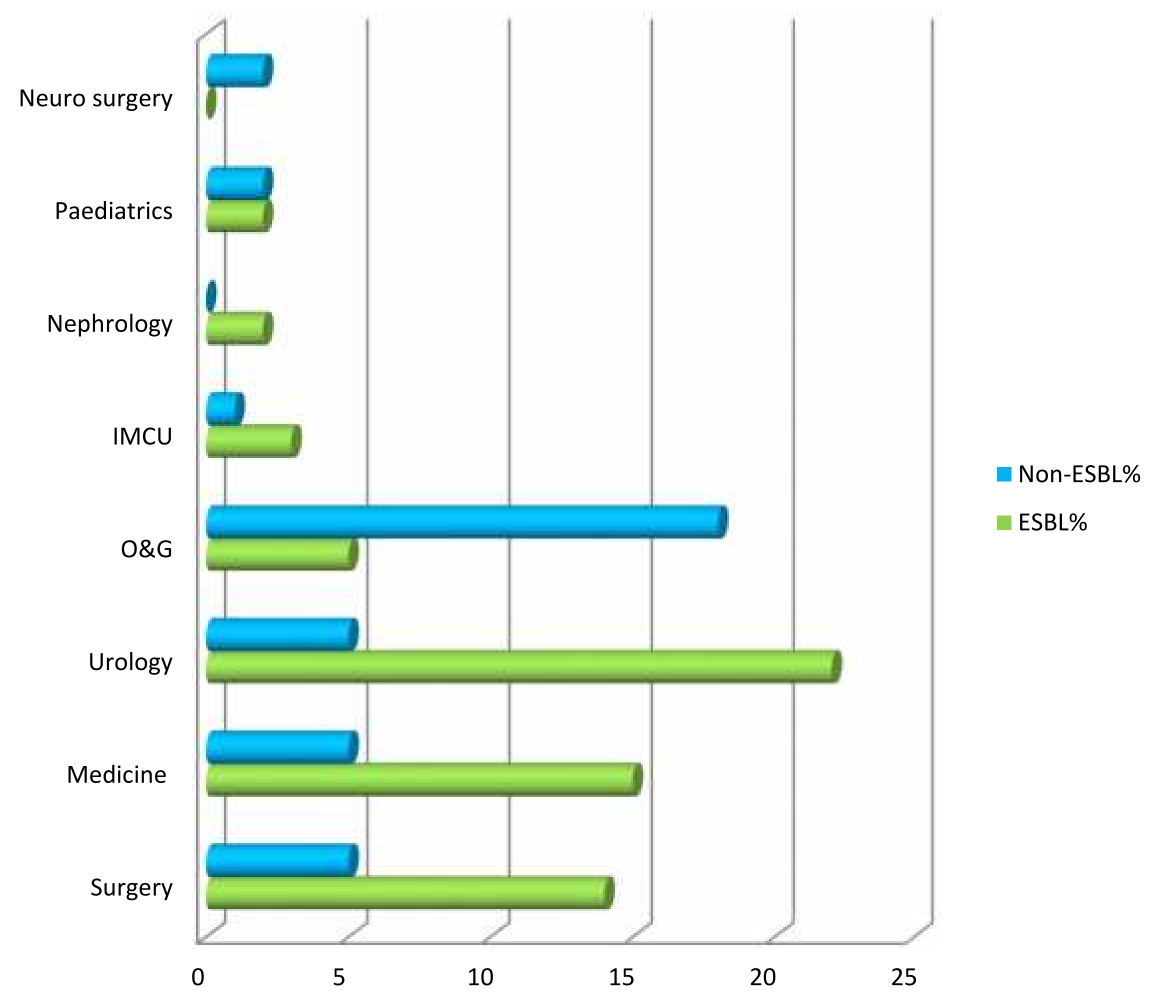
5.12 Distribution of ESBL isolates from wards

Table 15 Ward wise distribution of ESBL producing *E.Coli* isolates

Ward	**ESBL**		**Non-ESBL**	
	No	**%**	**No**	**%**
Surgery	14	22.22	5	13.51
Medicine	15	23.81	5	13.51
Urology	22	34.92	5	13.51
O&G	5	7.94	18	48.65
IMCU	3	4.76	1	2.70
Nephrology	2	3.17	-	-
Paediatrics	2	3.17	2	5.40
Neuro surgery	-	-	1	2.70
Total	**63**	**100**	**37**	**100**

The above table15,(Fig.5) shows the distribution of ESBL producing *Escherichia coli* samples from various departments of the hospital. Majority of the ESBL isolates were from Urology department 22 (34.92%) from the total of the 63 isolates. About 15 ESBL isolates (23.81%) were from Medicine department which contributes the second major cases. Fourteen isolates were from Surgery department. About 3 isolates were from IMCU and 2 isolates were from Nephrology and paediatric department respectively.

Fig. 5. **Distribution of ESBL isolates from wards**



5.13 Specimen wise distribution of ESBL isolates:

Table 16.Specimen wise distribution of ESBL and Non-ESBL isolates

Specimen	**ESBL**		**Non-ESBL**	
	No	**%**	**No**	**%**
Urine	41	65.08	25	67.57
Pus	17	26.98	5	13.51
Vaginal swab	1	1.59	4	10.81
Body fluids	2	3.17	1	2.70
Blood	1	1.59	1	2.70
Sputum	1	1.59	1	2.70
Total	**63**	**100**	**37**	**100**

Table 16.shows the categorizationof *Escherichia coli* on specimen basis. On analyzing various infections associated with *Escherichia coli*, 65.08%(n=41) of ESBL isolates were from urine, 26.98% (n=17) of ESBL isolates were from pus, 3.17% (n=2) were from ascitic fluid and one isolate each from vaginal swab, blood and sputum(1.59%).(Fig.6)

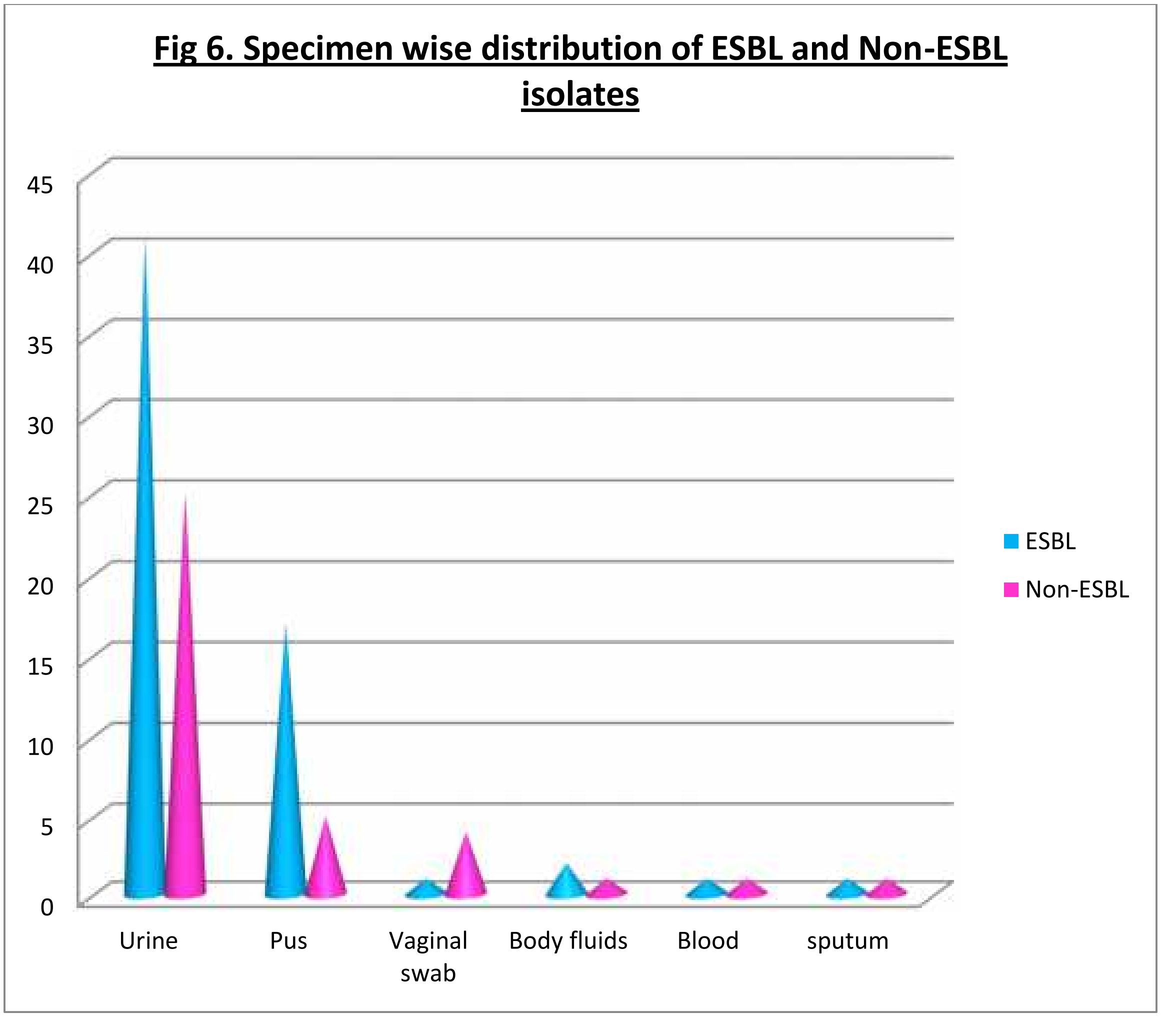


Fig 6. Specimen wise distribution of ESBL and Non-ESBL isolates
45
40
35
30
25
20
15
10
5
0
Urine
Pus
Vaginal swab
Body fluids
Blood
sputum
ESBL
Non-ESBL

<u>5.14 Categorization of ESBL and Non-ESBL *E.coli* on infection basis:</u>

Table.17 shows the categorization of *Escherichia coli* on infection basis. On analyzing various infections associated with ESBL producing *E.coli* urinary tract infections showed highest ESBL production of 65.08%(41/63*).Escherichia* About 12.70% of ESBL *E.coli* isolates were associated with wound infections, 14.3% of ESBL *E.coli* isolates were associated with diabetic foot (Fig.7)

Table:17 Categorization of ESBL and Non-ESBL *E.coli* on infection basis:

Infections	**ESBL**		**Non-ESBL**	
	No	**%**	**No**	**%**
Urinary tract infections	41	65.08	25	67.57
Diabetic foot	9	14.29	1	2.70
Wound infections	8	12.70	4	10.82
Respiratory tract infections	1	1.59	1	2.70
Vaginitis	1	1.59	4	10.81
Abdominal infections	2	3.17	1	2.70
Septicaemia	1	1.59	1	2.70
Total	**63**	**100**	**37**	**100**

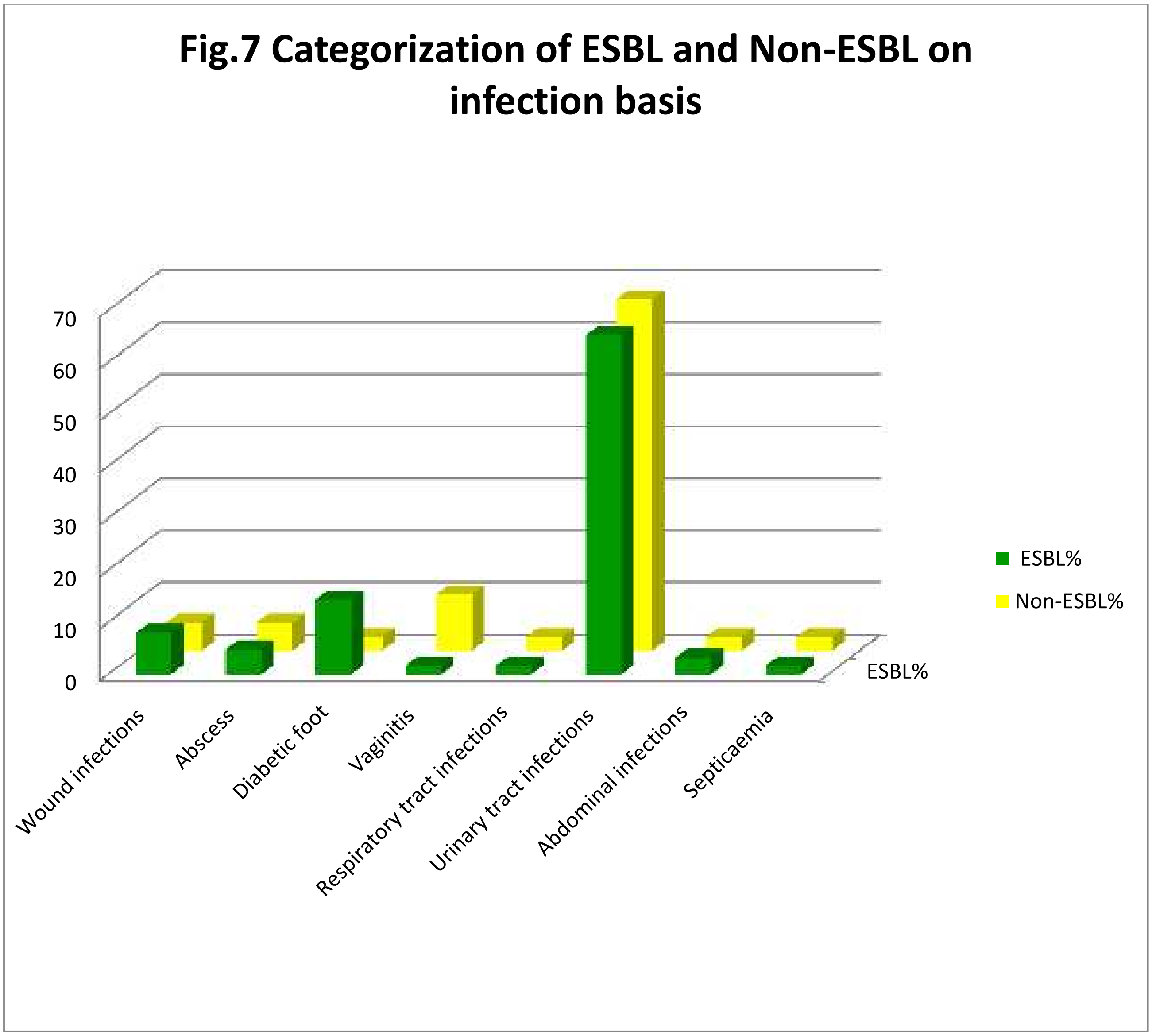


Fig.7 Categorization of ESBL and Non-ESBL on infection basis
70
60
50
40
30
20
10
0
ESBL%
Non-ESBL%
ESBL%
Wound infections
Abscess
Diabetic foot
Vaginitis
Respiratory tract infections
Urinary tract infections
Abdominal infections
Septicaemia

<u>5.15 Quinolone resistance among the *E.coli* isolates:</u>

Among the E.coli isolates 49 were found to be quinolone resistant and 5 were intermediate resistant by disc diffusion test using norfloxacin (10µg) and ciprofloxacin(5µg) disc. The zone of inhibition of ≤12 mm and ≤15mm for Norfloxacin and Ciprofloxacin respectively were taken as resistant. The zone of inhibition with 12-16mm and 16-20 mm were taken as intermediate resistant. A total of 49 isolates and 5 isolates were quinolone resistant and intermediate resistant to quinolone respectively. Both the resistant strains and intermediate strains were subjected to MIC determination by E test. E- test confirmed all the 49 resistant strains as quinolone resistance and in addition identified additional two strains as quinolone resistance.(Table 18)

Table 18.Detection of quinolone resistance by phenotypic methods

. Method	Quinolone resistance	
	No	%
Disc diffusion test	49	49
E-Test	51	51

<u>5.16 Coexistence of Quinolone Resistance and ESBL in *E.coli* isolates</u>

Table 19.coexistence of quinolone resistance in ESBL *E.coli*

	ESBL		Non –ESBL		Total
	No	%	No	%	
Quinolone resistance	43	68.25	8	21.62	51
Quinolone sensitive	20	31.75	29	78.38	49
	63	100	37	100	100

The above table 19, shows quinolone resistant among ESBL and non-ESBL producing *E.coli* isolates. Of the 63 ESBL producing E.coli, 43(68.25%) were resistant to quinolones. And among the 43 non-ESBL *E.coli* isolates 8(21.62%) were resistant to quinolones. Coexistence of quinolone resistance among the ESBL producing *E.coli* isolates were stastically significant by Chi-square test(p-value<0.0001)

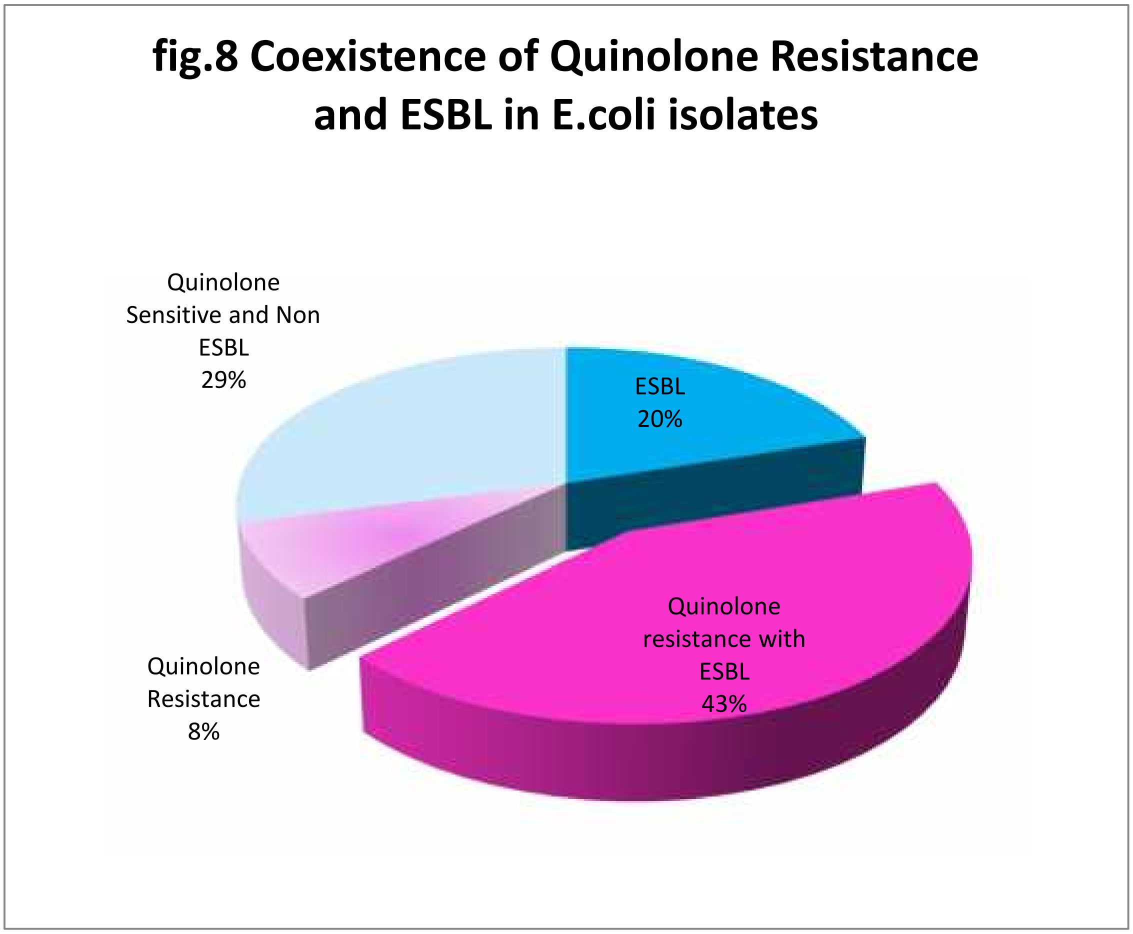


fig.8 Coexistence of Quinolone Resistance and ESBL in E.coli isolates
Quinolone Sensitive and Non ESBL 29%
ESBL 20%
Quinolone resistance with ESBL 43%
Quinolone Resistance 8%

<u>5.17 Specimen wise distribution of Coexistence of quinolone resistance and ESBL production among *E.coli* isolates:</u>

Table 20 Specimen wise distribution of quinolone resistance among ESBL *E.coli*

N=43

Specimen	**No**	**%**
Urine	29	67.44
Pus	10	23.26
Ascitic Fluid	2	4.65
Blood	1	2.32
Sputum	1	2.32
	43	100

The specimen wise distribution of quinolone resistant ESBL producing *E.coli* were shown in table 20. Among these urine 29 (67.44%) showed highest percentage of positive isolates. The isolation rate among other specimens were pus10(23.26%), ascitic fluid 2(4.65%) and blood and sputum each with 1 isolates(2.32%)

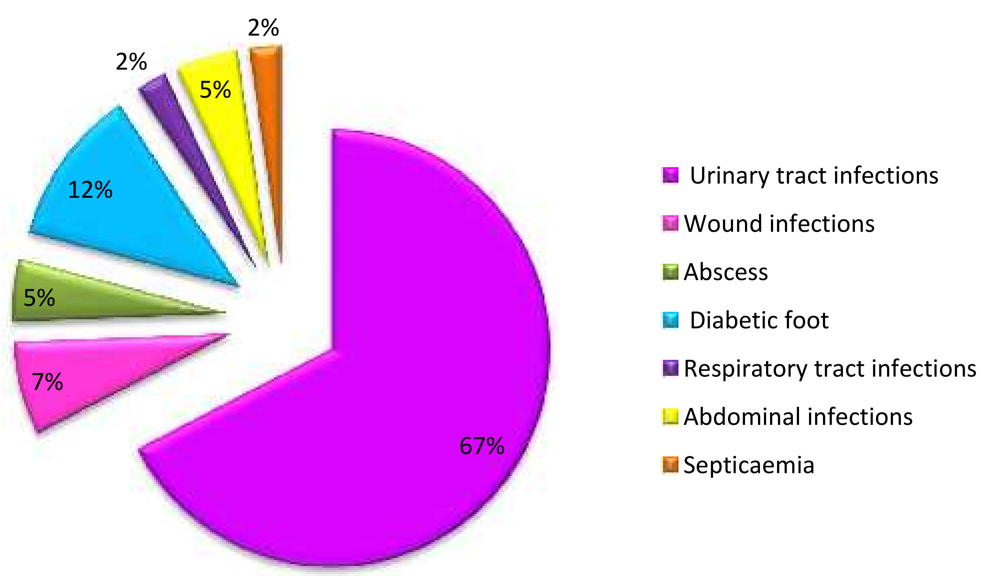
5.18 Categorization of quinolone resistant ESBL*E.coli* on infection basis:

Table 21. Distribution of the quinolone resistant ESBL producing *E.coli* among various infections

Infections	**Quinolone resistance and ESBL production**	
	No	**%**
Urinary tract infections	29	67.44
Wound infections	3	6.98
Abscess	2	4.65
Diabetic foot	5	11.63
Respiratory tract infections	1	2.33
Abdominal infections	2	4.65
Septicaemia	1	2.33
Total	**43**	**100**

The above table.21 shows the categorization of *E.coli* with quinolone resistant ESBL producing strains on infection basis. The majority of quinolone resistant ESBL isolates were assosited with urinary tract infections 29(67.44%) . 11.63% of isolates were associated with diabetic foot.. about 4.65% of isolates were associated with abcesses and abdominal infections

Fig 9.Distribution of the quinolone resistant ESBL producing *E.coli* among various infections

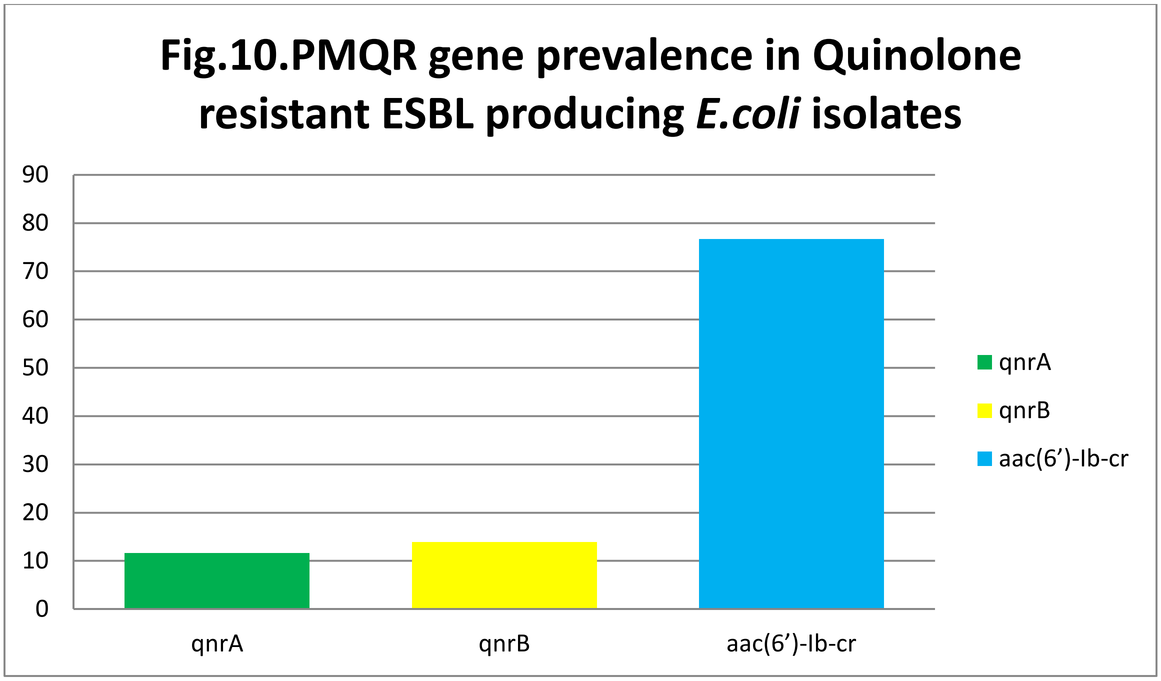


5.19 Detection of Plasmid mediated quinolone resistance genes among quinolone resistance strains :

Table 22. Detection of PMQR by real time PCR.

Gene amplified	No of isolates	Percentage %
aac(6')-Ib-cr	22	51
qnrA and aac(6')-Ib-cr	5	11.7
qnrB and aac(6')-Ib-cr	6	14
Not amplified	10	23.3
Total	43	100

Of th**e** 43 quinolone resistant ESBL producing isolates, 22(51%) isolates harbored *aac-(6')-Ibr* gene and 6(14%) isolates possessed both qnrB and *aac-(6')-Ib-cr gene ; qnrA* and *aac-(6')-Ib-cr gene* were present in 5(11.7%) isolates. Ten isolates were did not possess any of the three gene. In total 33 isolates 76.74% of ESBL producing quinolone resistance harbored *aac-(6')-Ib-cr* gene and 11 had *qnr* gene (5 q*nrA* gene and 6 *qnrB* gene).(Table.22,Fig.10)



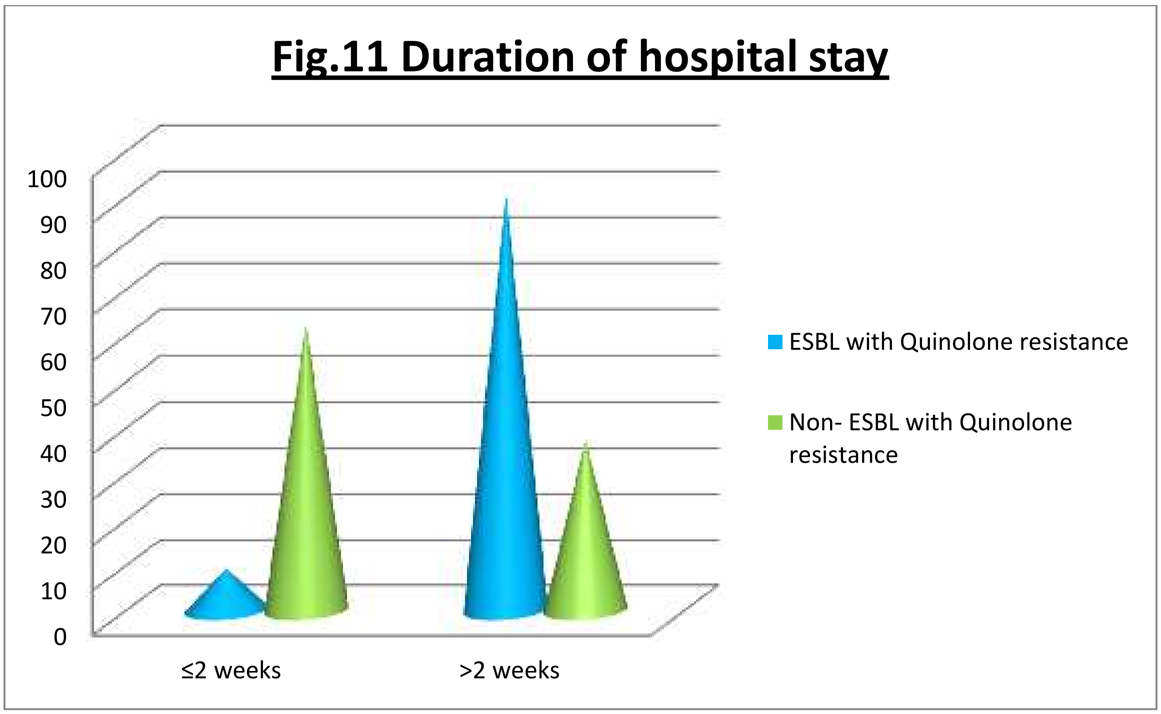
5.20. Analysis of Risk Factors:

5.20.1. Duration of hospital stay:

About 90.7% of the ESBL with quinolone resistance-*E.coli* isolates were obtained from the patients with more than 2 weeks of hospital stay. Only 9.3% of the Non-ESBL with quinolone resistance isolates were from the patients with >2 weeks of hospital stay. The association of ESBL with quinolone resistance-*E.coli* isolates with the duration of hospital stay was statistically significant. P < 0.0001 by Chi-square test (Table.23& fig.11)

Table: 23. Duration of hospital stay

Duration in days	ESBL with Quinolone resistance		Non –ESBL with Quinolone resistance	
	No	%	No	%
≤2 weeks	4	9.3	5	62.5
>2 weeks	39	90.7	3	37.5
Total	**43**	**100**	**8**	**100**



5.20.2. Exposure to antibiotics:

Table:24. Exposure to antibiotics

Antibiotics	**ESBL With quinolone resistance N=43**	**Non-ESBL With quinolone Resistance N=8**	**Significance $p<0.05$**
Cephalosporins	23	2	Significant
Aminoglycosides	19	1	Significant
Fluoroquinolones	26	3	Significant
Cotrimoxazole	4	2	Non –Significant
Doxycycline	1	1	Non –Significant

There was statistically significant difference in exposure to cephalosporins, quinolones and Aminoglycosides among quinolone resistant ESBL and Quinolone sensitive ESBL isolates (P<0.05). There is no significance in the administration of other mentioned antibiotics with the corresponding quinolone resistant ESBL and quinolone sensitive ESBL isolates.Table.24.(Fig: 11)

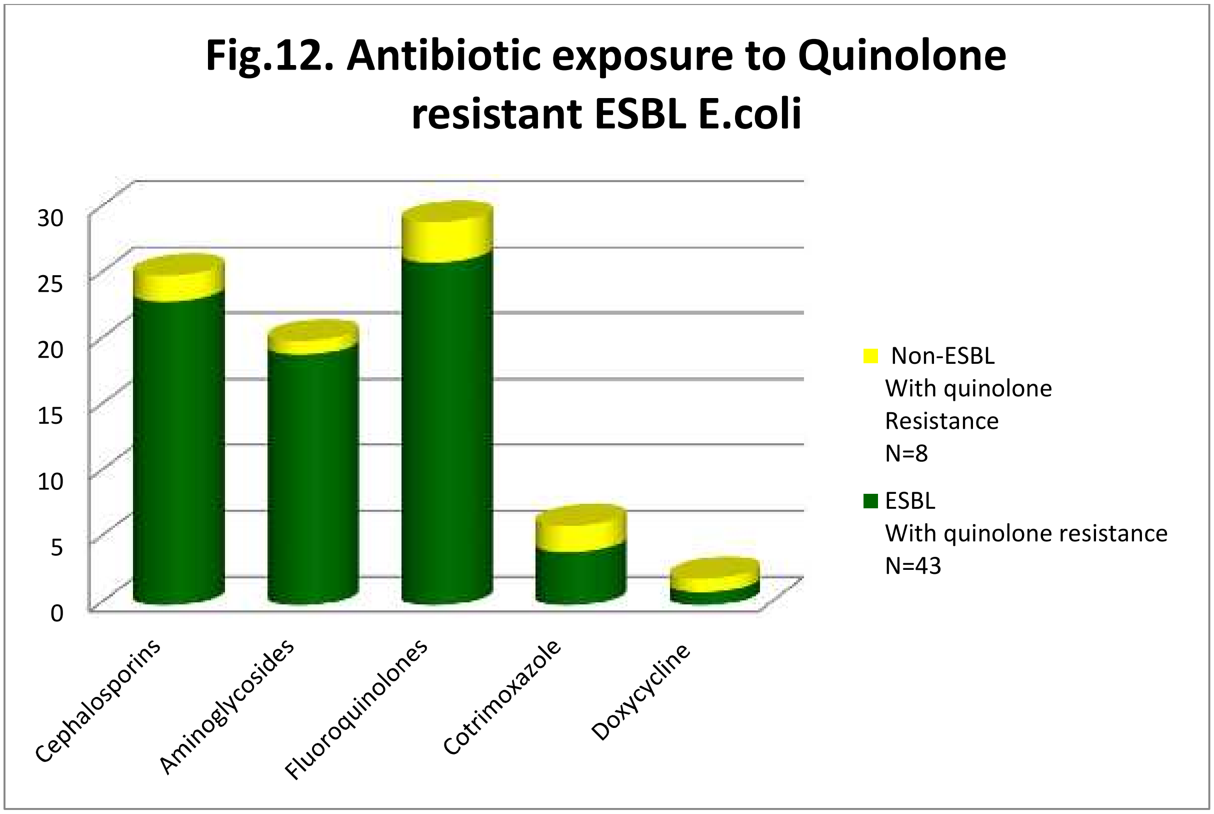


Fig.12. Antibiotic exposure to Quinolone resistant ESBL E.coli
30
25
20
15
10
5
0
Cephalosporins
Aminoglycosides
Fluoroquinolones
Cotrimoxazole
Doxycycline
Non-ESBL With quinolone Resistance N=8
ESBL With quinolone resistance N=43

6. DISCUSSION

Escherichia coli is one of the leading cause for the hospital acquired infections among Enterobacteriaceae. *Escherichia coli* exhibit wide degree of resistance to various antimicrobials. It shows acquired resistance, more commonly to beta lactams, quinolones and aminoglycosides. Multidrug resistance among *Escherichia coli* is steadily increasing. The rise in the multi-drug resistance has become a major challenge in treating the *E.coli* infections.

Several mechanisms are responsible for the acquired resistance to the β lactam antibiotics in *E.coli* which includes the production of β lactamases, up regulation of the efflux pump systems and decreased outer membrane permeability. With respect to β lactamases, the extended spectrum beta lactamases are the most prevalent beta lactamases in the Enterobacteriaceae family especially among *E.coli.* The genes coding beta lactamases are plasmid coded. These plasmids also have genes resistant to other antibiotics.

The most common extra intestinal clinical manifestation of *E.coli* is the urinary tract infection and the Quinolones are the most commonly prescribed antibiotics for both prophylaxis and treatment purposes as it is available in the oral formulations also. Thus there is an increase in the prevalence of quinolone resistance. The quinolone resistance is both chromosomal mediated and plasmid mediated. The plasmid mediated resistance gene were carried along with the ESBL. The close relationship between ESBL production and quinolone resistance is worrisome as the first reported instance of plasmid-mediated ciprofloxacin resistance was isolated in an isolate of *Klebsiella pneumoniae* also possessed an ESBL.

The present study was aimed to identify the prevalence of quinolone resistant ESBL producing *Escherichia coli* from various specimens in the Department of Microbiology, Tirunelveli medical college.

6.1. Age – Sex wise distribution of the Study group:

The present study showed that the *E.coli* was isolated more in the age group of 41-70 years (64%). This was in correlation with the study conducted by Chakraborty et al from Karnataka during 2013 in which the isolation of *E.coli* was 72% in the age group of 41-70 years[79] and Shristi Raut et al who showed that 49.9% of *E.coli* were from the patients in age group of 41-70 years at a tertiary care hospital, Nepal during 2015[80].

In the present study *E.coli* was isolated more commonly in females (73%) when compared to males (27%) with a male: female ratio of 1:2.7. A similar observation was made by Tarchouna et al[81] in Tunisia, during 2015 in which *E.coli* was isolated more in females, with the male to female ratio as 1:3. Unlike the present study, Chakraborty et al in his study showed that the male: female ratio was 1.2: 1 where the male: female ratio was relatively equal[79].

6.2. Distribution of *Escherichia coli* in wards:

The rate of isolation of *E.coli* in this study was highest in the urology wards (27%) followed by O&G (23%), medicine wards (20%) and surgery ward(19%). *E.coli* was isolated less from paediatrics ward, IMCU and nephrology ward. Raut et al noted majority of *E.coli* isolated from various wards when compared to IMCU, this is in accordance with this study[80]. The study by Chakraborty et al showed maximum number of *E.coli* isolates were from medical unit followed by surgical ward, urology ward, O&G ward this is in contrast to this study, however similar to this study less number of isolates were recovered from paediatrics units[79]. The increased rate of isolation in urology ward may be because, this study was carried in a tertiary care hospital with well established speciality wards like urology ward.

6.3. Distribution of *Escherichia coli* in clinical isolates:

Most of the specimens collected in this study were from urine(66%), followed by pus and wound swab (22%), vaginal swab (5%), body fluids(3%)and 2% from blood and sputum. This was in correlation with the study conducted by Kibret et al in which *E.coli* was most commonly isolated from urine(45.22%) followed by pus(22%), aspirate (11%) and blood(8%)[82]. A study by Sarathkumar Shetty et al also showed that *E.coli* were most commonly isolated from the urine and pus samples (74.6%)[83]. Zahra Shahandeh et al in their study also showed that E.coli were most commonly isolated from urine(80%), and about(3.4%) from body fluids, similar to this study[84].

6.4. Resistance pattern of *Escherichia coli* isolates to antibiotics:

The overall antibiotic sensitivity pattern of *E.coli* in our study showed higher sensitivity to Imipenem(96%) and Piperacillin-tazobactam(92%). There was higher degree of resistance towards Ampicillin and Cotrimoxazole (72%) followed by cephalosporins and Ciprofloxacin(49%). The urine samples were also tested with nitrfurantoin.This was in accordance with the study of Dinesh kumar et al where the

E.coli showed a higher degree of sensitivity towards Imipenem and Piperacillin-Tazobactam[85]. In this study *E.coli* showed 43% and 33% of resistance towards Amikacin and Gentamicin respectively. This is similar to the study of Pooja patel et al where the *E.coli* isolates showed a resistance of about 40% and 32% respectively[86].

6.5. Screening for ESBL production :

In our study ESBL screening was done using Cefotaxime 30µg disc, ceftriaxone 30µg disc and ceftazidime 30 µg disc as per CLSI guidelines which recommends use of more than one drug for increased sensitivity of screening method. Among the cephalosporins used for screening Ceftazidime has better sensitivity followed by Ceftriaxone. Out of 70 screening positive isolates, Ceftazidime detected 63(90%) isolates as potential ESBL producers followed by Ceftriaxone which detected 59(84.29%) isolates as ESBL producers whereas Cefotaxime detected 56 isolates(78.57%).

This was similar to the study conducted by Giriyapur et al who showed the sensitivity of about 89.9%, 79.83% and 86.72% for Ceftazidime, Cefotaxime and Ceftriaxone discs in sceening for ESBL respectively[87]. Ho PL et al in the study about ESBL revealed sensitivities of 57.7% , 98.6% 99.3% for Ceftazidime, Ceftriaxone and Cefpodoxime respectively for screening tests using Kirby Baeur disc diffusion method[71].

6.6 Phenotypic detection of ESBL*E.coli.*

In our study, ESBL production was confirmed with Combined Disc Diffusion Test(CDDT), Double Disc Synergy Test(DDST), and ESBL E-test. Out of the 70 isolates which were positive with screening procedure 63 were confirmed as ESBL producers. Isolates with positive result in any one of the three confirmatory test were considered positive for ESBL production. As CLSI guidelines recommends CDDT as one of the confirmatory test, in this study CDDT was considered as the standard phenotypic confirmatory test for ESBL detection.

Among the 70 screening positive isolates 60(85.71%). were confirmed with CDDT. Similar results were seen in a study done by Suryawanshi et al where 87.91%(168/191) of screening positives were ESBL producers by CDDT[88]. However Anilchander et al in their study showed that CDDT detected 38.70% of ESBL screening positive isolates[89]. This is in contrast with the present study results. The prevalence rate in the above study is low when compared to the present study.That may be due to difference in population size or sample size and also the health care practices followed in those areas.

In our study, DDST confirmed 54 isolates as ESBL producers among the 70 isolates positive with the screening test. The sensitivity, specificity, positive predictive value, negative predictive value of the DDST with reference to CDDT were 89%,90%,98% and 57% respectively. The sensitivity and specificity of the DDST varies in various studies. This lack of sensitivity results from the fact that DDST is not a standardized procedure[87]. In a study in Andhrapradesh, India by Giriyapur et al, DDST had a sensitivity of 94.89% (167/176), a specificity of 75.91% (104/137), and a positive predictive value of 83.55% (167/200) and negative predictive value of 92.03%[87]. The sensitivity of DDST varies with the distance between the clavulanate containing disc and the third generation cephalosporin containing disc.(14,15) Ho PL *et al*. in their study reported the sensitivity of DDST to be 83.8% at a interdisc distance of 30 mm in DDST[71] . They also reported that the sensitivity can be increased to 97.9% by decreasing the distance between the cephalosporin disc and clavulanate-cephalosporin disc to 20 mm. Vercauteren *et al.*, reported sensitivities of the double disc synergy test to be 96.9%. Sensitivity of the DDST in this study is less, this may be due to the decreased activity of ESBL[90](esbl update ref 409)

Out of 70 screening positive isolates 61(87.14%) were confirmed to produce ESBL with E-test. In our study E-test detected maximum cases when compared to CDDT and DDST. E-test had the sensitivity of 96.6% and 70% specificity. The positive predictive value and negative predictive value were 95% and 77.7% respectively with reference to CDDT, Wiegand et al in their study reported sensitivity and specificity of 98.6% and 72.7% respectively for which is in agreement with the present study results[91].

Gaurav dalelal et al described that E-test ESBL strip results showed a highly significant correlation with PCDDT similar to this study[92].

Sasirekha et al in their study reported that out of the 145 ESBL screen positive isolates, 110 isolates were ESBL producers by the ESBL E-test strip. MIC values of ESBL positive isolates for cefotaxime alone were ≥ 64 μg/ml and for cefotaxime/clavulanate combination was ≥ 1 μg/ml.

In addition the ESBL E test has the advantage over the other phenotypic tests, in that the minimum inhibitory concentration can be obtained which will be useful in the treatment of resistant pathogens. Despite the accuracy, the ESBL E test is very costly to be used in the clinical laboratory for routine ESBL screening procedure. Due to the cost constraints of the ESBL E test a simple and cost effective method should adopted.

Among the screening positive isolates 7 were not confirmed by any of the three methods. This could be due to some other resistance mechanism other than ESBL production, such as AmpC production.

According to this study 63% of *E.coli* were confirmed as ESBL producers. This is study correlated with the study by Jaspal Gaur *et al*., who identified a prevalence rate of 63.4 % from four different tertiary care hospitals from across India[93] and the study by Ankur Goyal *et al*., from NewDelhi, who reported a prevalence rate of 63.6%[94].The study by Gururajan et al., showed 47% of ESBL producing *E.coli*[95] . This is lower than the isolation of ESBL *E.coli* in our study. Other study by Maya et al showed a higher prevalence of ESBL *E.coli* (75.5%)[96].

6.8 Distribution of ESBL *E.coli* isolates according to age and gender

In our study ESBL were more commonly isolated from females,

(33) than males (30). This is in similarity to the studies by Mulvey et al in which ESBL were more common among females with male:female ratio of about 32:37[62]. Another study by Chakraborty et al, showed that ESBL were more commonlyisolated from males(54%) than from females(46%)[79]. The same study showed that ESBL were more common in the age group >45 years(73%). This is in coincident with our study, with ESBL more commonly isolated from the age group > 45 years.

6.9 Distribution of ESBL *E.coli* isolates in different specimens

Most of the ESBL *E.coli* isolates in this study *E.coli* were from urine (65.08%) followed by pus (26.08%), body fluids (3.17%) and blood(1.59%). A study by Sarker et al[97], and Umadevi et al[98] showed that *E.coli* were mostly isolated from the urine samples (59-64%) followed by pus. While the present study showed the isolation of *E.coli* were more in urine there are other studies that showed the isolation was highest in specimens like pus (Rudhresh et al [53]) .

6.10 Distribution of ESBL- *E.coli* in wards:

The rate of isolation of ESBL *E.coli* in this study was highest in urology wards wards (34.92%) followed by medicine (23.81%) and surgery wards (22.22%). A similar observation was made in a study by Ankur Goyal et al[94]. Chakraborty et al in their study observed ESBL producing *E.coli* more in medical ward(46%) followed by surgery(14%) and urology ward (15%)[79].

6.11 Distribution of ESBL *E.coli* isolates according to site of infection:

In the present study, 65.08% ESBL producing *E.coli* isolates were associated with urinary tract infections, 14.29% of the isolates were from diabetic foot and 12.70% from wound infection and abcesses, 3.17% were associated with ascites and 1.59% were from bacteremia.

The study by Chakraborty et al showed that ESBL-*E.coli* was predominantly isolated from urinary tract infections(51.5%)[79]. Fourteen per cent of the isolates were obtained from various wound infections. This is in concordence with our study. However

Chakraborty et al observed ESBL in about 26.5% of bacteremia which is very high than our study.

6.12 Quinolone resistance among *E.coli* isolates :

In our study among the E.coli isolates 49 were found to be quinolone resistant and 5 were intermediate resistant by disc diffusion test. Both the resistant strains and intermediate strains were subjected to MIC determination by E test. E- test confirmed all the 49 resistant strains as quinolone resistance and in addition identified two strains as quinolone resistance. Quinolone resistant *E.coli* was 51% in this study. This is comparable to the study by Cavaco et al, which confirmed that low level resistance was best detected by MIC testing[99].

6.13 Coexistence of quinolone resistant ESBL producing *E.coli*:

In this study of the 63 ESBL producing *E.coli* infections, 43(68.25%) were fluroquinolone resistant and 20 were sensitive to fluroquinolones(31.75%). This is comparable with the study done by Hassan et al at hospitals in the Cairo,Egypt in which among 30 ESBL *E.coli* 13(60%) were resistant to quinolone[100]. In another study by Iraj Pakzad et al of 42 ESBL producing E.coli 24 (64.2%) were resistant to Quinolones[74]. This is in accordance with our result. However Philippe et al from Manitoba in 2007 found that 79.4% of ESBL-positive *E.coli* isolates was resistant to Quinolones[101].

6.14 Prevalence of plasmid mediated quinolone resistance genes among the quinolone resistant ESBL producing *E.coli*:

Out of 43 quinolone resistant ESBL producing *E.coli* strains, 33(76.4%) carried *aac-(6')-Ib-cr* gene. The *qnrA* and *qnrB* were detected in 5 and 6 isolates respectively.

The prevalence of *aac-(6')-Ib-cr* gene in our study was found to be high. This is similar to the study by Yugendran et al,2015[100], at JIIPMER, Pudhucherry. In this study among 642 quinolone resistant isolates 414 (64.5%) carried *aac-(6')-Ib-cr* gene. Another study by Pazhani et al showed a prevalence of 64.6% of *aac-(6')-Ib-cr* among enteric *E.coli* isolates[101]. Yang et al, in 2014, at a tertiary care center in Korea concluded that *aac-(6')-Ib-cr* gene was detected in 73.8%(59/80) of quinolone resistant *E.coli* isolates, similar to our study[102]. However in other studies the prevalence of *aac-(6')-Ib-cr* ranged between 7% to 40%.(yang pasom veldman).

The prevalence of qnr gene in our study was 25.5%. qnrA was detected in 5 isolates and qnrB in 6 isolates.

A study by Taruchouna et al.,showed that among the qnr gene, qnrB were predominant than qnrA. This is comparable to our study, qnrB were more common than qnrA. Conversely Iraj pakzad et al in their study observed among the 24 *E.coli* producing ESBLs and resistant to ciprofloxacin, 37.5%(n=9) and 20.8%(n=4) harboured qnrA and qnrB, respectively.

The 10 isolates that did not possess any of the three gene may be because, it may possess other plasmid genes which were not included in this study such as qnrS, qnrD or it may be due to other mechanism of quinolone resistance like loss of porin channels, alteration in the membrane permeability.

6.15. Analysis of risk factors:

There was no significance difference in the mean age, sex ratio among the patients with Quinolone resistant ESBL producing *E.coli* and Quinolone sensitive ESBL producing *E.coli* strains. Exposure to different classes of antibiotics in the patients harbouring Quinolone resistant ESBL *E.coli* and Quinolone sensitive *E.coli* strains were analysed. Among these, exposure to antibiotics like third generation cephalosporins, aminoglycosides, fluoroquinolones, β lactamase + β lactamase inhibitor combinations and carbapenems, exposure to third generation cephalosporins, fluoroquinolones and aminoglycosides were statistically significant among corresponding quinolone resistant in infections caused by ESBL *E.coli.*

In this study, 90.7% strains of ESBL producing Quinolone resistant were from patients with more than two weeks stay in hospital .This is similar to the study by Lautenbach et al in which long term stay in hospital was a risk factor for quinolone resistance in infections due to ESBL producing *E.coli.* Lautenbach et al in their study evaluated various risk factors for Fluroquinolone resistance in infections due to ESBL producing *E.coli and Klebsiella pneumoniae.* In their study they concluded that prior fluroquinolone use, aminoglycoside use and long term stay at hospitals were independent risk factor for quinolone resistance in infections due to ESBL *E.coli.* A study by Rooney et al in their study concluded that prior quinolone use, history of urinary tract infection were associated with quinolone resistant in ESBL producing *E.coli.*

7.SUMMARY

- In the present study the isolation of *E.coli* were more in the age group of 41-70 years.
- The present study showed infections due to *E.coli* more in females (69%) than males (31%) with a male: female ratio of 1:2.2.
- The rate of isolation of *E.coli* was highest in Urology wards (27%).
- Urine was the most common specimen accounting for 66% followed by pus 22%, vaginal swab 5%, and blood 2%.
- About 63% of the *E.coli* isolates were found to be ESBL producers.
- Majority of the ESBL producers were associated with urinary tract infections (65.08%).
- The combined disc diffusion test (CDDT) using Cefotaxime and Ceftazidime with and without clavulanic acid detected 60(95.23%) isolates of screening positive ESBL producer.
- The double disc synergy test(DDST) detected about 85.71% of the resistant isolates as ESBL producers and the sensitivity, specificity, positive predictive value and negative predictive value of DDST for detecting ESBL were 84%, 90%, 98% and 56.2% respectively.
- The ESBL E test in this study detected 96.83% of the resistant isolates as ESBL producer and the sensitivity, specificity, positive predictive value and negative predictive value were 97%, 70%, 95% and 78% respectively.

- The CDDT is the simple, cost effective method in the detection of the ESBL and can be used routinely in the laboratory for the detection of ESBL.
- The E test is relatively accurate in the detection of ESBL however it is costly to use routinely in the clinical laboratory.
- Quinolone resistance among the *E.coli* isolates were 51%. Disc diffusion test detected 96% of quinolone resistance.
- Co-existence of quinolone resistance among the ESBL producing *E.coli* were 68.25 %(43/63). Coexistence of quinolone resistance among ESBL producing *E.coli* was statistically significant with p value, 0.0001.
- Among the quinolone resistant ESBL producing *E.coli* isolates, plasmid mediated quinolone resistance genes *qnrA*, *qnrB* and *aac-(6')-Ib-cr* were detected in 33 isolates (76.74%) by PCR. Among the 33 isolates 6 isolates harboured both *qnrB* and *aac-(6')-Ib-cr* and 5 isolates possed both *qnrB* and *aac-(6')-Ib-cr* gene.
- Duration of stay at hospital for more than two weeks and prior use of antibiotics such as Cephalosporins, and Quinolones had statistically significant association with quinolone resistant ESBL producing *E.coli*.($P < 0.05$)
- Quinolone resistant ESBL producing *E.coli* isolates showed 95.43% sensitive to imipenam and 88.37% sensitive to piperacillin- tazobactam.

8. CONCLUSION

- This study highlights the prevalence of ESBL among various clinical isolates and also detects the coexistence of quinolone resistance among ESBL *E.coli.*
- The CDDT and ESBL E test are very accurate in detecting the ESBL. However ESBL-E test has the cost constraints. Combined disc diffusion test is very simple, easy and cost effective that can be easily adopted in the clinical laboratory. Early detection of the ESBL is crucial to include timely execution of strict infection control practices and treatment with alternative antimicrobials and to further prevent the transmission of coresistance of other drugs especially the fluoroquinolones as it has broad spectrum activity with orally available drugs.
- Good antibiotic policy, judicial use of antibiotics, adequte dosing to limit mutant selection, hand hygiene and continuous monitoring of antibiotic resistance, will fight against the serious therapeutic challenges due to the infections caused by these resistant *E.coli*.
- The carbapenems are the drug of choice for ESBL producing quinolone resistant *E.coli* strains. However carbapenem resistance are emerging worldwide. Colistin has become the last resort drug that can be used for the multidrug resistant *Enterobacteriacae*.

ANNEXURE – 1

1. Nutrient agar medium:

Composition

Ingredients gram/liter

Peptic digest of Animal Tissue 5.00

Sodium Chloride 5.00

Beef Extract 1.50

Yeast Extract 1.50

Agar 15.00

Twenty-eight grams of dehydrated nutrient agar medium was added to 1000 ml of cold distilled water in a flask and boiled to dissolve the medium completely. The medium was then sterilized in an autoclave at 121^0C and 15 lbs pressure for 15 minutes. The sterile media were stored in a refrigerator at 4^0C for future use.

2. MacConkey agar medium:

Composition - Ingredients gram/liter

Peptone 19.0

Lactose 10.0

NaCl 5.0

Na- Deoxycholate 1.0

Neutral Red 0.03

Crystal Violet 0.001

Agar 15.0

Fifty-two grams of dehydrated MacConkey agar medium was suspended in 1000 ml of cold distilled water and boiled to dissolve the medium completely. The solution was then sterilized by autoclaving at 121^0C and 15 lbs pressure for 15 minutes.

3. Blood agar medium

Composition

Ingredients gram/liter

Heart infusion 500.00

Tryptose 10.00

Sodium chloride 5.00

Agar 15.00

Forty grams of the dehydrated blood agar medium was suspended in 1000 ml cold distilled water in a flask and boiled to dissolve the medium completely. It was then sterilized by autoclaving at 121^0C and 15 lbs pressure for 15 minutes. The autoclaved materials were allowed to cool to a temperature of 45^0C in a water bath. Defibrinated 5-10% sheep blood was then added to the medium aseptically and distributed to sterile petri dishes. Sterile media was stored in refrigerator at 4^0C for future use.

4. Muller Hinton agar medium

Composition

Ingredients gram/liter

Beef dehytrated infusion 300

Casein hydrolysate 17.50

Starch agar 17.00

Agar 17.00

Thirty-eight grams of dehydrated Mueller Hinton agar medium was suspended in 1000 ml cold distilled water and boiled to dissolve the medium completely. The solution was then sterilized by autoclaving at 121^0C and 15 lbs pressure for 15 minutes. The autoclaved media was stored in the refrigerator and used later.

5. McFarland Standard (0.5):

Reagents:

Sulphuric acid, 1%: To 100 ml of distilled water,1 ml of conc.sulphuric acid is added. Barium chloride, 1.175%: To 100 ml of distilled water, 1.175gm of barium chloride is added and mixed well.

To prepare McFarland 0.5 standards:

To 85 ml of 1% conc.sulphuric acid, 0.5 ml of Barium chloride is added in a flask while constantly swirling the flask. Bring to 100 ml with 1% conc.sulphuric acid. Aliquot in test tubes and cap tubes tightly. Store in the dark at room temperature for 3 months or longer.

Interpretation of Antibiotic susceptibility testing

Antibiotic	**Concentration in** μg	**Sensitive**	**Intermediate**	**Resistant**
Ceftriaxone	30	>23	20-22	<19
Cefotaxime	30	>26	23-25	<22
Ceftazidime	30	>18	15-17	<14
Gentamicin	10	>15	13-14	<12
Amikacin	30	>17	15-16	<14
Ciprofloxacin	5	>21	16-20	<15
Norfloxacin	5	>17	14-16	<13
Imipenem	10	>19	16-18	<15
Pip – Tazo	100	>21	15-20	<14

PROFORMA

Name :

Age :

Sex :

OP/IP No :

Lab No :

Ward :

Complaints :

Clinical diagnosis :

Nature of Specimen :

Duration of hospital stay :

Antibiotics administered :

Investigation :

Biochemical tests : Indole, Citrate, Urease, Triple sugar iron,

Catalase, Oxidase

Antibiogram : Penicillin, Amikacin, Ceftriaxone, Cefotaxime, Ceftazidime, Ciprofloxacin, Co-trimoxazole, Norfloxacin, Gentamicin, Imipenam, Piperacillin with Tazobactam.

ESBL Screening test with Disc Diffusion test

Combined disc diffusion test

Double disc synergy test

E test

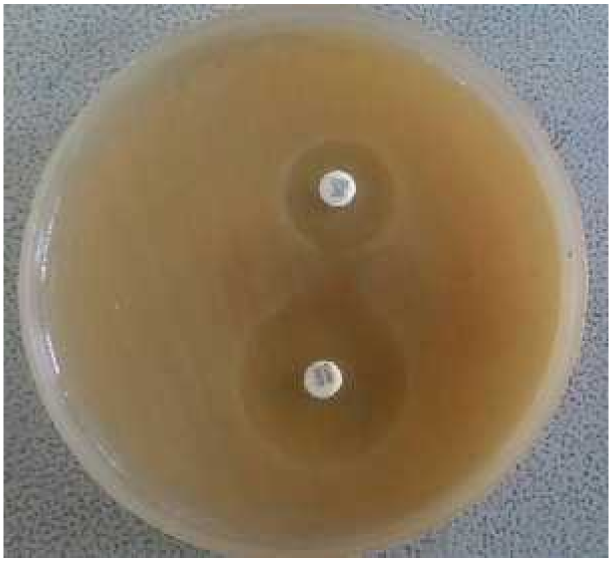
Quinolone resistance- E-test

RT-PCR(PMQR) gene

COLOUR PLATE 2

A. COMBINED DISC DIFFUSION TEST

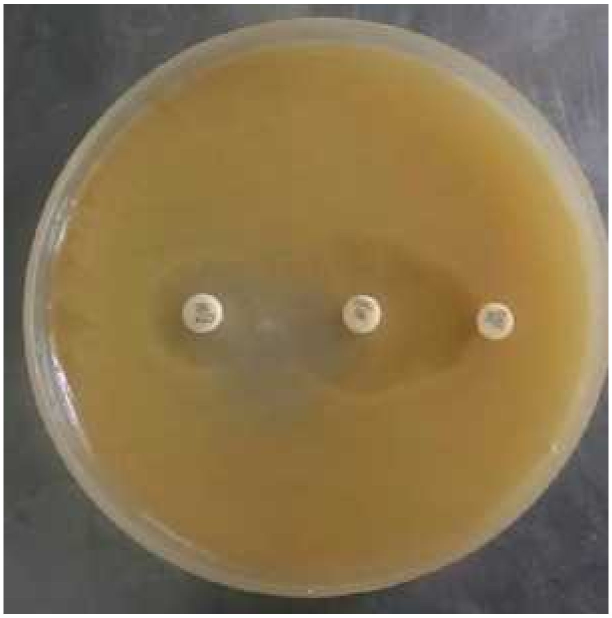
ESBL Producer



B.DOUBLE DISC SYNERGY TEST

Enhancement of zone of inhibition towards Amoxicillin–Clavulunate disc

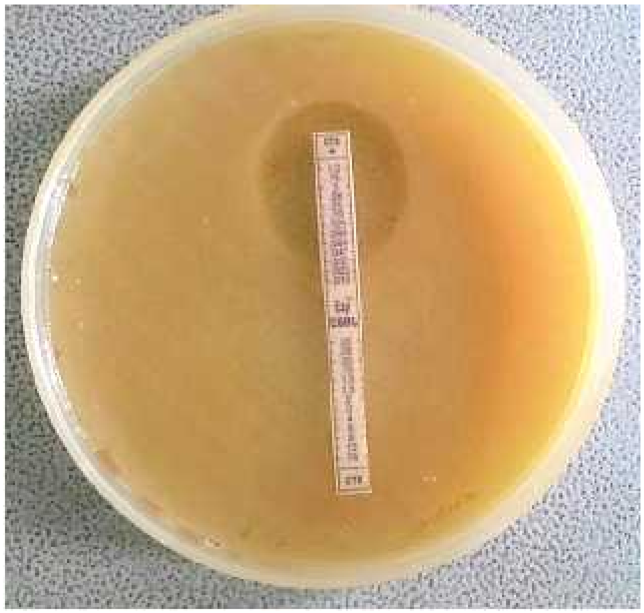
ESBL Produced by DDST



COLOUR PLATE 3

ESBL E-Test

A. ESBL Producer



B. Non-ESBL Producer

COLOUR PLATE 4

A. DISC DIFFUSION TEST- Quinolone Resistance

B. Quinolone Sensitive

COLOUR PLATE 5

E-Test

A. Quinolone Resistance

B. Quinolone Sensitive

COLOUR PLATE 6

Molecular Characterization by PCR

Thermocycler

COLOUR PLATE 7

A. DNA Extraction Kit

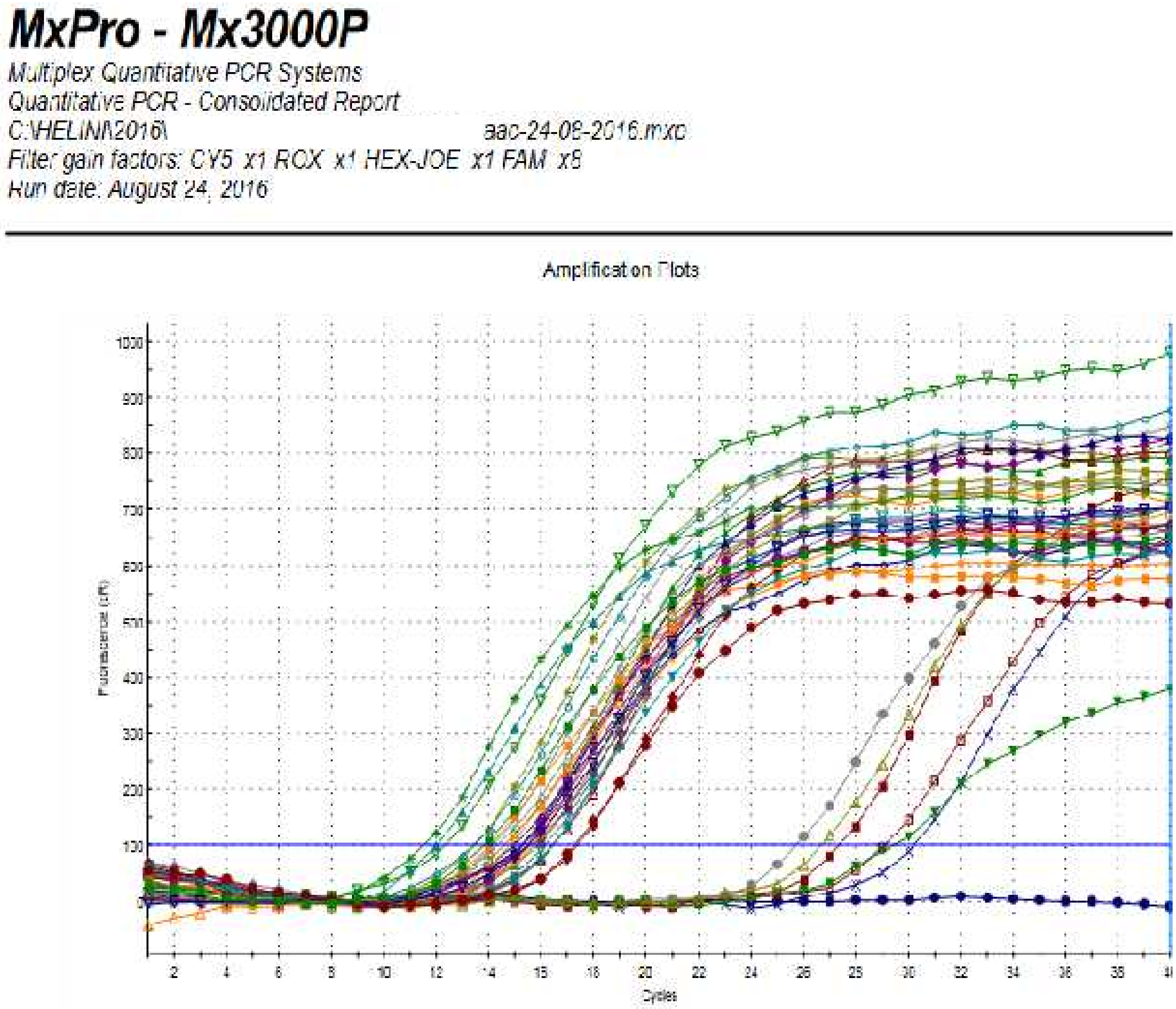
B.DNA Extraction

COLOUR PLATE-8

PCR Amplification Kit

COLOUR PLATE 9

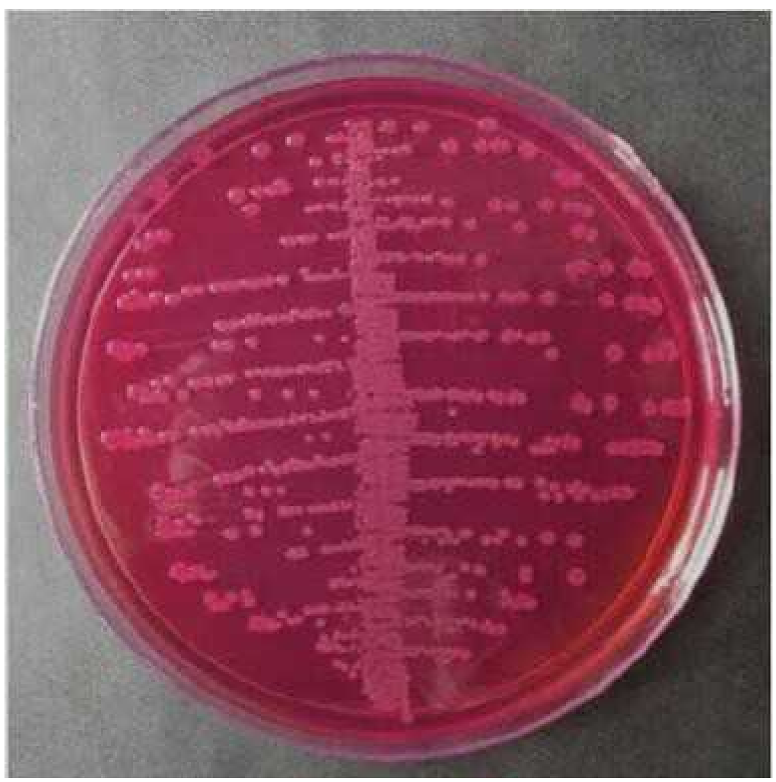
Real Time- PCR- AMPLIFICATION CHART



COLOUR PLATE -1

A.Nutrient agar plate showing translucent colonies

B. Mac Conkey Agar plate showing lactose fermenting colonies



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